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(57) Abstract

The present invention is directed to recombinant hosts expressing novel proteins associated with Alzheimer's Disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. This invention is specifically directed to the recombinant hosts and vectors which contain the genes coding for the neuronal thread proteins. This invention is also directed to substantially pure neural thread protein, immunodiagnostic and molecular diagnostic methods to detect the presence of neural thread proteins, and the use of nucleic acid sequences which code for neural thread proteins in gene therapy.

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Neural Thread Protein Gene Expression and Detection of Alzheimer's Disease

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

The present invention was made with U.S. government support. Therefore, the U.S. government has certain rights in the invention.

Cross Reference to Related Applications

This is a continuation-in-part of U.S. Application Serial No. 08/050,559, filed April 20, 1993, the contents of which are fully incorporated by reference herein.

Field of the Invention

The present invention is in the field of genetic engineering and molecular biology. This invention is directed to recombinant hosts expressing novel proteins associated with Alzheimer's Disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. This invention is specifically directed to the recombinant hosts and vectors which contain the genes coding for the neuronal thread proteins. This invention is also directed to substantially pure neural thread proteins, immunodiagnostic and molecular diagnostic methods to detect the presence of neural thread proteins, and the use of nucleic acid sequences which code for neural thread proteins in gene therapy.

Background of the Invention

Alzheimer's Disease

Alzheimer's Disease (AD) is the most frequent cause of dementia in the United States, affecting over two million individuals each year. It is a degenerative brain disorder characterized clinically by loss of memory, confusion, and gradual physical deterioration. It is the fourth most common cause of death. The etiology of the disease is virtually unknown but has been attributed to various viruses, toxins, heavy metals, as well as genetic defects. The disease is at present incurable.

Until quite recently, AD was thought to account for relatively few of the cases generally classified as senile dementia. Other factors can lead to such a condition, including repetitious mild strokes, thyroid disorders, alcoholism, and deficiencies of certain vitamins, many of which are potentially treatable. It can be appreciated, then, that a diagnostic test specific for AD would be very useful for the clinical diagnosis and proper clinical treatment of subjects presenting with symptoms common to all of these conditions.

The brains of individuals with AD exhibit characteristic pathological accumulations of congophilic fibrous material which occurs as neurofibrillary tangles within neuronal cell bodies, and neuritic (or senile) plaques. Neurofibrillary tangles may also be found in the walls of certain cerebral blood vessels. The major organized structural components of neurofibrillary tangles are paired helical filaments. Qualitatively indistinguishable amyloid deposits also occur in normal aged brains but in much smaller numbers with restricted topographical distribution.

There has been considerable recent investigative activity regarding the characterization of proteins found in neuritic plaques and neurofibrillary tangles of AD and other neurologic diseases. One of the amyloid proteins initially described by Glenner *et al.* has been cloned and sequenced (Glenner *et al., Biochem. Biophys. Res. Commun. 120*:1131-1135 (1984); U.S. Patent No. 4,666,829). The A4 amyloid protein found in neuritic plaques and blood

vessels has been determined to be a component of a 695 amino acid precursor; a protein postulated to function as a glycosylated cell surface receptor (Masters *et al., Proc. Natl. Acad. Sci. USA 82*:4245-4249 (1985), Kang *et al.*, *Nature 325*:733-736 (1987)). In addition, the amyloid protein has been postulated to function as a cell adhesion molecule and as a calcium ion channel protein (Hooper, *J. NIH Res. 4*: 48-54 (1992); Rensberger, *Wayward Protein Molecule May Be Elusive Killer of Brain Cells*, *The Washington Post*, January 25, 1993, §1, at A3 (1993)). The gene coding for A4 is located on chromosome 21 (Kang *et al.*, *ibid.*; Goldgaber *et al.*, *Science 235*:877-880 (1987); Tanzi *et al.*, *Science 235*:880-885 (1987); St. George-Hyslop *et al.*, *Science 235*:885-889 (1987)) but apparently is not linked to the familial form of the disease (Van Broekhoven *et al., Nature 329*:153-155 (1987)). There appears to be little, if any, protein sequence homology between amyloid A4 and ß protein, their higher molecular weight precursor, and pancreatic thread protein (PTP) (Gross *et al., J. Clin. Invest. 76*:2115-2126 (1985)).

A number of other proteins thought to be associated with the disease have been described, including Ubiquitin, ALZ-50, microtubular-associated proteins τ and MAP2, and neurofilament protein (*see*, for example, Manetto *et al., Proc. Natl. Acad. Sci. USA 85*:4502-4505 (1988); Wolozin *et al.*, *Science 232*:648-651 (1986); Selkoe, *Neurobiol. Aging 7*:425-432 (1986); Perry *et al.*, in: *Alterations of the Neuronal Cytoskeleton in Alzheimer's Disease*, Plenum, New York, pp 137-149 (1987)). More recently, a serine protease inhibitor called α_1-anti-chymotrypsin has been found in AD amyloid deposits (Abraham *et al., Cell 52*:487-501 (1988)).

There is currently no useful diagnostic test for AD being practiced clinically. A definitive diagnosis is possible only postmortem, or during life through a brain biopsy, to reveal the presence of the characteristic plaques, tangles, paired helical filaments, and other cerebrovascular deposits which characterize the disorder. Such an invasive surgical procedure is inherently dangerous and is therefore rarely utilized. As a result, the clinical misdiagnosis of AD is estimated to be approximately 20%-30%.

Thread Proteins

The prototype thread protein molecule is pancreatic thread protein (PTP) which bears the unusual physical property of forming insoluble fibrils at neutral pH, but is highly soluble at acid or alkaline pH (Gross *et al.*, *supra*). PTP is highly abundant, synthesized by pancreatic acinar cells, and secreted into pancreatic juice in concentrations exceeding 1 mg/ml (*Id.*). An increased thread protein immunoreactivity has been demonstrated in brains with AD lesions, using monoclonal antibodies to PTP (Ozturk *et al.*, *Proc. Natl. Acad. Sci. USA 86*:419-423 (1989)). In addition, a highly sensitive forward sandwich immunoradiometric assay was used to demonstrate that at least three distinct antigenic epitopes were shared between PTP and the related protein in the brain (*Id.*) Despite similarities, the pancreatic and neuronal forms of the thread protein are almost certainly distinct since the mRNA molecules and proteins differ in size, and many of the antigenic epitopes which are present in the pancreatic thread protein are not detectable in brain tissue (de la Monte *et. al.*, *J. Clin. Invest. 86*:1004-1013 (1990); de la Monte *et. al.*, *J. Neurol. Sci. 113*:152-164 (1992); de la Monte *et al.*, *Ann. Neurol. 32*:733-742 (1992)).

The central nervous system form of the thread protein, designated hereafter as "neural thread protein" (NTP). has been identified in AD and Down's Syndrome brain tissue (Wands *et al.*, International Application Publication No. WO 90/06993). NTP has been found in all AD brains studied where characteristic neuropathologic changes of the disease exist (*Id.*). The saline- extractable soluble immunoreactivity shares has a molecular weight of approximately 17 to 20 kD (*Id.*).

Quantitative measurements of NTP immunoreactivity in various regions of AD brains revealed levels varying from 12 to 295 ng/gm tissue (Mean = 116 ng/gm tissue) compared to 1-11 ng/gm tissue (Mean = 5 ng/gm tissue) in comparable ares of control brains (*Id.*).

Immunocytochemistry performed with monoclonal antibodies directed against the pancreatic form of PTP demonstrated that NTP is localized within

cells, within fine processes within the neuropil, or is extracellular in both AD and Down's Syndrome brains (*Id.*). Two types of cell contain NTP: neurons and astrocytes (*Id.*). The affected neurons are the large pyramidal type which typically contain the neurofibrillary tangles well known in AD brain (*Id.*).

That NTP accumulation within neurons is intrinsically important or integrally related to the evolution of AD lesions is corroborated by the presence of identical patterns of immunolabeling for NTP in Down's Syndrome brains, but not in control brains (*Id.*). It is important to note that the same structural abnormalities of AD occur in brains of all middle-age individuals with Down's syndrome, whether or not they are demented. There is also a higher incidence of AD in family members of Down's Syndrome patients. Moreover, the regional differences in the densities of NTP-containing neurons parallels the density distributions of neurofibrillary tangles in both AD and Down's Syndrome. This provides further evidence that NTP is germane to the pathophysiology of AD. Whether NTP accumulates within neuronal perikarya, as a result of aberrant cellular metabolism or transport is not yet known.

Summary of the Invention

A need exists for a definitive diagnostic test which can be performed on individuals suspected of having, or being at risk for AD. The present invention satisfies such needs and provides further advantages.

The manner in which these and other objects are realized by the present invention will be apparent from the summary and detailed description set forth below.

Unless defined otherwise, various terms used herein have the same meaning as is well understood in the art to which the invention belongs. All cited publications are incorporated herein by reference.

This invention is directed to recombinant hosts expressing novel proteins associated with Alzheimer's Disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. This invention is specifically directed to the recombinant hosts and vectors which contain the genes coding for the neuronal thread proteins (NTP) having molecular weights of about 8 kDa, 14 kDa, 17 kDa, 21 kDa, 26 kDa or 42 kDa. This invention is also directed to the substantially pure neural thread proteins, immunodiagnostic and molecular diagnostic methods to detect the presence of neural thread proteins, and the use of nucleic acid sequences which code for neural thread proteins in gene therapy.

In particular, the invention includes a method for detecting and quantitating an NTP in a human subject, comprising:

(a) contacting a biological sample from a human subject that is suspected of containing detectable levels of an NTP with a molecule capable of binding to the NTP; and

(b) detecting the molecule bound to the NTP.

The invention additionally includes the method as above, wherein the binding molecule is selected from the group consisting of:

(a) an antibody substantially free of natural impurities;

(b) a monoclonal antibody; and

(c) a fragment of (a) or (b).

The invention additionally includes the method as above, wherein the detecting molecule is detectably labeled and where a combination of such binding molecules is used.

The invention additionally includes a method for detecting the presence of a genetic sequence coding for an NTP in a biological sample using a polynucleotide probe derived from a recombinant human NTP of this invention.

The invention additionally includes a method for determining the presence of a condition in a human subject, said condition including, but not limited to, the group consisting of Alzheimer's Disease, the presence of neuroectodermal tumors, the presence of malignant astrocytomas, and the presence of gliomas.

The invention additionally includes a method of diagnosing the presence of AD in a human subject suspected of having AD which comprises:

(a) incubating a biological sample from said subject suspected of containing an NTP with a molecule capable of identifying an NTP; and

(b) detecting the molecule which is bound in the sample, wherein the detection indicates that the subject has AD.

The invention additionally includes a method of diagnosing the presence of neuroectodermal tumors in a human subject suspected of having neuroectodermal tumors which comprises:

(a) incubating a biological sample from said subject suspected of containing an NTP with a molecule capable of identifying an NTP; and

(b) detecting the molecule which is bound in the sample, wherein the detection indicates that the subject has neuroectodermal tumors.

The invention additionally includes a method of diagnosing the presence of a malignant astrocytoma in a human subject suspected of having a malignant astrocytoma which comprises:

(a) incubating a biological sample from said subject, which is suspected of containing an NTP, in the presence of a binding molecule capable of identifying an NTP; and

(b) detecting molecule which is bound in the sample, wherein the detection indicates that the subject has a malignant astrocytoma.

The invention additionally includes a method of diagnosing the presence of a glioblastoma in a human subject suspected of having a glioblastoma which comprises:

(a) incubating a biological sample from said subject, which is suspected of containing an NTP, in the presence of a binding molecule capable of identifying an NTP; and

(b) detecting molecule which is bound in the sample, wherein the detection indicates that the subject has a glioblastoma.

The invention additionally includes the methods as above, wherein a biological sample is removed a human subject prior to contacting the sample with the molecule.

The invention additionally includes the methods as above, wherein detecting any of the molecules bound to the protein is performed by *in situ* imaging.

The invention additionally includes the methods as above, wherein detecting of any of the molecule bound to the protein is performed by *in vivo* imaging.

The invention additionally includes the methods as above, wherein the biological sample is reacted with the binding molecule in a manner and under such conditions sufficient to determine the presence and the distribution of the protein.

The invention additionally includes the methods as above, wherein a detectably labeled binding molecule of an NTP is administered to a human subject.

The invention additionally includes the methods as above, wherein the binding molecule is bound to the protein *in vivo*.

The invention additionally involves an NTP substantially free of any natural impurities and having a molecular weight of about 42 kDa.

The invention additionally involves an NTP substantially free of any natural impurities and having a molecular weight of about 26 kDa.

The invention additionally includes an NTP substantially free of any natural impurities and having a molecular weight of about 21 kDa.

The invention additionally includes an NTP substantially free of any natural impurities and having a molecular weight of about 17 kDa.

The invention additionally includes an NTP substantially free of any natural impurities and having a molecular weight of about 14 kDa.

The invention additionally includes an NTP substantially free of any natural impurities and having a molecular weight of about 8 kDa.

The present invention also particularly relates to the diagnostic methods recited above, wherein the immunoassay comprises two different antibodies bound to a solid phase support combined with a third different detectably labeled antibody in solution.

The invention is also directed to a method of producing an NTP, said method comprising:

(a) culturing a recombinant host comprising a human gene coding for said NTP; and

(b) isolating said NTP from said host.

Additionally, the invention is directed to a substantially pure NTP obtained by the such a process.

The invention is also directed to an 15- to 30-mer antisense oligonucleotide which is complementary to an NTP nucleic acid sequence and which is nonhomologous to PTP nucleic acid sequences, as well as pharmaceutical compositions comprising such oligonucleotides and a pharmaceutically acceptable carrier.

The invention is also directed to ribozymes comprising a target sequence which is complementary to an NTP sequence and nonhomologous to PTP nucleic acid sequences, as well as pharmaceutical compositions comprising such ribozymes and a pharmaceutically acceptable carrier.

The invention is also directed to a method of achieving pharmaceutical delivery of NTP molecules to the brain through acceptable carriers or expression vectors.

The invention is also directed to oligodeoxynucleotides that form triple stranded regions with the various NTP genes (nucleic acid sequences) and

which are nonhomologous to PTP nucleic acid sequences, as well as pharmaceutical compositions comprising such oligodeoxynucleotides and a pharmaceutically acceptable carrier.

The invention is also directed to the therapeutic use of NTP-derived molecules or fragments thereof to modify or improve dementias of the Alzheimer's type of neuronal degeneration.

The invention is also directed to methods for the differential diagnosis of sporadic and familial Alzheimer's disease.

Brief Description of the Drawings

Figure 1 (panels A-J) shows neural thread protein immunoreactivity in CNS-derived tumors.

Figure 2 depicts a graph showing neural thread protein levels in PNET1, PNET2, A172, C6, and Huh7 hepatocellular carcinoma cells measured by a forward sandwich monoclonal antibody-based immunoradiometric assay (M-IRMA).

Figure 3 shows molecular size of neural thread proteins in SH-Sy5y, A172, and C6 cells demonstrated by immunoprecipitation and Western blot analysis using the Th9 monoclonal antibody.

Figure 4 shows molecular sizes of neural thread proteins in PNET1 cells (a) and C6 glioblastoma cells (b) demonstrated by pulse-chase metabolic labeling with ^{35}S-methionine, and immunoprecipitation with Th9 monoclonal antibody (left panel). The molecular weights are 8, 14, 17, 21, 26 and 42 kDa (arrows).

Figure 5 depicts a series of five graphs showing the 21 kDa and 17 kDa neural thread proteins in SH-Sy5y, PNET1, A172, and C6 cells and the absence thereof in Huh7 cells by SDS-PAGE/M-IRMA.

Figure 6 depicts a gel showing that the 21 kDa neural thread protein in C6 glioblastoma cells is phosphorylated.

Figure 7 depicts a bar graph showing altered neural thread protein expression in PNET1 cells with growth phase.

Figure 8 (panels A-F) shows altered phenotype of PNET1 cells with cessation of cell growth and overnight serum starvation.

Figure 9 shows the 1-9a partial cDNA sequence, and Figure 9a shows a partial sequence of the second 5′ anchor PCR product corresponding to the 5′ region of the 1-9a cDNA (WP5′ Sequence).

Figure 10 shows alignment of partial sequences between 1-9a and human PTP and the Reg gene (the nucleic acid sequence corresponding to the genomic clone of human PTP).

Figure 10a shows alignment between 1-9a and Exon 2 of the human Reg gene, and between the first 5′ anchor PCR product of 1-9a (WP03-417) and Exon 2 and Reg.

Figure 10b shows alignment between the 1-9a and its second 5′ anchor PCR product (WP5′) and AD 3-4 and AD 2-2 cDNAs.

Figure 11 shows the partial nucleic acid and deduced amino acid sequences of the HB4 cDNA as well as a protein hydrophilicity window plot.

Figure 11a shows alignment between HB4 and human PTP.

Figure 11b shows alignment between HB4 and human Reg gene.

Figure 12 (panels A-C) shows the expression of mRNA molecules corresponding to the 1-9a CNS neural thread protein cDNA sequence in neuroectodermal tumor cell lines and in rat pancreas.

Figure 13 (panels A and B) shows mRNA transcripts corresponding to the 1-9a CNS neural thread protein cDNA sequence in human brain. This figure also demonstrates higher levels of 1-9a CNS neural thread protein-related mRNAs in AD brains compared with aged-matched controls (panel A). Panel B demonstrates four different transcripts with greater abundance of the lower molecular size mRNAs in AD compared with aged controls.

Figure 14 (panels A-C) shows 1-9a Southern blot analysis of RT/PCR-derived cDNAs in neuroectodermal cell lines. A- and B-PCR amplification of 1-9a mRNA sequences in neuroectodermal cell lines, and using mRNA

from newborn rat (NB) brain, AD brain, and aged control brain. Panel A is a longer exposure of Panel B. Panel C shows hybridization of the same blot using the O18 rat PTP probe.

Figure 15 (panels A and B) (SE-RT/PCR) shows hybridization of the 1-9a and O18 probes with several clones isolated from SH-Sy5y cells by reverse transcribing mRNA and amplifying with primers corresponding to the known sequence of the 1-9a partial cDNA.

Figures 16, 16a and 16b show the partial nucleic acid sequences of the AD 2-2 cDNAs isolated from the AD brain library. Figure 16 also shows a hydrophilicity window plot of AD2-2 T7.

Figures 16c, 16d, 16e and 16f show the partial nucleic acid sequences of the AD 3-4 cDNAs isolated from the AD brain library. Figure 16c also shows a hydrophilicity window plot of AD3-4.

Figures 16g, 16h and 16i show the partial nucleic acid sequences of the AD 4-4 cDNAs isolated from the AD brain library.

Figure 16j shows the partial nucleic acid sequences of the AD 16c (also called AD 10-7) cDNAs isolated from the AD brain library. Figure 16j also shows a hydrophilicity window plot of AD16c-T7.

Figure 16k shows the complete nucleotide sequence of the AD10-7 cDNA clone that was isolated from an AD library.

Figure 16l shows the complete nucleotide sequence of the AD16c cDNA clone that was isolated from the AD brain library.

Figure 17 shows alignment of partial sequences between AD 2-2 and human Reg gene.

Figure 17a shows alignment of partial sequences between AD 2-2 and Exon 1 of Reg and rat PTP.

Figure 17b shows alignment of partial sequences between AD 2-2 and 1-9a.

Figure 17c shows alignment of partial sequences between AD 2-2 and AD 16c.

Figure 18 shows alignment of partial sequences between AD 3-4 (also called AD 5-3) and the Reg gene.

Figure 18a shows alignment of partial sequences between AD 3-4 and the 5' anchor PCR products of the 1-9a mRNA, termed WPO3-5 and 18-4.

Figure 18b shows alignment of partial sequences between AD 3-4 and the G2a-a *Eco*RI/*Pst*I genomic clone.

Figure 19 shows alignment of partial sequences between AD 4-4 and AD 2-2 and 1-9a (also called SE-4 corresponding to the PCR clone which is identical to 1-9a).

Figure 20 shows alignment of partial sequences between AD 16c and Reg gene.

Figure 20a shows alignment of partial sequences between AD 16c and human PTP.

Figure 20b shows alignment of partial sequences between AD 16c and AD 2-2.

Figure 21 (panel A) shows a genomic Southern blot analysis using the AD 3-4 as a probe; Figure 21 (panel B) shows a similar pattern of hybridization on a genomic Southern using AD 2-2 as a probe. Figure 21 (panel C) shows a Northern blot analysis of neuroectodermal tumor cell lines using AD 3-4 as a probe. The four cell lines that exhibit AD 3-4 transcripts are neuronal in phenotype; C6 glioma cell mRNA did not hybridize with the AD 3-4 probe. Figure 21 (panel D) shows a Northern analysis of human AD and aged control brain temporal lobe tissue using the AD 3-4 probe, and demonstrates over-expression of the corresponding gene in AD (lanes labeled A) compared with aged control brains (lanes labeled C).

Figures 22, 22a, 22b, 22c, 22d, 22e, 22f, 22g and 22h shows partial sequences of four genomic clones (isolated using both the 1-9a cDNA and rat PTP O-18 cDNA as probes.

Figures 23 and 23a show the alignment of the G2a-2 *Pst*I partial sequence with the Reg gene.

Figure 23b shows alignment of the G2a-2 *Pst*I-*Eco*RI sequence and the Reg gene and the rat PTP.

Figures 23c and 23d show the alignment of the G5d-1 *Pst*I sequence and the Reg gene.

Figure 24 shows neural thread protein expression by the 1-9a cDNA (panel A) and the G2a-2 *Pst*I genomic clone (panel B). Panels C and D show negative expression by the G5d-1 *Eco*RI/*Pst*I genomic clone, and pBluescript which lacks a cloned insert, respectively.

Figure 25 depicts a Northern blot analysis of AD16c mRNA in AD and aged control brains. The data shows elevated levels of AD16c mRNA expression in 6 of 9 AD compared to 1 of 6 age-matched controls.

Figure 26 depicts a Western blot analysis of AD10-7 fusion proteins using monoclonal antibodies against the expressed tag protein (T7-tag mouse monoclonal antibodies.

Figure 27 (panels A and B) depicts brightfield and darkfield microscopic analysis of the *in situ* hybridization of sense and antisense cRNA probes to human brain tissue sections of early AD.

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cloning vector. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in

the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive or inducible.

Substantially pure. As used herein means that the desired purified protein is essentially free from contaminating cellular components, said components being associated with the desired protein in nature, as evidenced by a single band following polyacrylamide-sodium dodecyl sulfate gel electrophoresis. Contaminating cellular components may include, but are not limited to, proteinaceous, carbohydrate, or lipid impurities.

The term "substantially pure" is further meant to describe a molecule which is homogeneous by one or more purity or homogeneity characteristics used by those of skill in the art. For example, a substantially pure NTP will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: molecular weight, chromatographic migration, amino acid composition, amino acid sequence, blocked or unblocked N-terminus, HPLC elution profile, biological activity, and other such parameters. The term, however, is not meant to exclude artificial or synthetic mixtures of the factor with other compounds. In addition, the term is not meant to exclude NTP fusion proteins isolated from a recombinant host.

Recombinant Host. According to the invention, a recombinant host may be any prokaryotic or eukaryotic cell which contains the desired cloned genes on an expression vector or cloning vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the desired gene(s) in the chromosome or genome of that organism.

Recombinant vector. Any cloning vector or expression vector which contains the desired cloned gene(s).

Host. Any prokaryotic or eukaryotic cell that is the recipient of a replicable expression vector or cloning vector. A "host," as the term is used herein, also includes prokaryotic or eukaryotic cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, *see* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

Promoter. A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Gene. A DNA sequence that contains information needed for expressing a polypeptide or protein.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Antisense RNA gene/Antisense RNA. In eukaryotes, mRNA is transcribed by RNA polymerase II. However, it is also known that one may construct a gene containing a RNA polymerase II template wherein a RNA sequence is transcribed which has a sequence complementary to that of a specific mRNA but is not normally translated. Such a gene construct is herein termed an "antisense RNA gene" and such a RNA transcript is termed an "antisense RNA." Antisense RNAs are not normally translatable due to the presence of translation stop codons in the antisense RNA sequence.

Antisense oligonucleotide. A DNA or RNA molecule containing a nucleotide sequence which is complementary to that of a specific mRNA. An

antisense oligonucleotide binds to the complementary sequence in a specific mRNA and inhibits translation of the mRNA.

Antisense Therapy. A method of treatment wherein antisense oligonucleotides are administered to a patient in order to inhibit the expression of the corresponding protein.

Complementary DNA (cDNA). A "complementary DNA," or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Homologous/Nonhomologous Two nucleic acid molecules are considered to be "homologous" if their nucleotide sequences share a similarity of greater than 50%, as determined by HASH-coding algorithms (Wilber, W.J. and Lipman, D.J., *Proc. Natl. Acad. Sci. 80*:726-730 (1983)). Two nucleic acid molecules are considered to be "nonhomologous" if their nucleotide sequences share a similarity of less than 50%.

Ribozyme. A ribozyme is an RNA molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, and self-cleaving RNAs.

Ribozyme Therapy. A method of treatment wherein ribozyme is administered to a patient in order to inhibit the translation of the target mRNA.

Fragment. A "fragment" of a molecule such as NTP is meant to refer to any polypeptide subset of that molecule.

Functional Derivative. The term "functional derivatives" is intended to include the "variants," "analogues," or "chemical derivatives" of the molecule. A "variant" of a molecule such as NTP is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analogue" of a molecule such as NTP is meant to

refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Examples of moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980) and will be apparent to those of ordinary skill in the art.

NTP. The term "NTP" refers to a family of neural thread proteins. The NTP family includes proteins with molecular weights of about 8 kDa, 14 kDa, 17 kDa, 21 kDa, 26 kDa and 42 kDa, as described herein.

Immuno-Polymerase Chain Reaction. A method for the detection of antigens using specific antibody-DNA conjugates. According to this method, a linker molecule with bispecific binding affinity for DNA and antibodies is used to attach a DNA molecule specifically to an antigen-antibody complex. As a result, a specific antigen-antibody-DNA conjugate is formed. The attached DNA can be amplified by the polymerase chain reaction (PCR) using appropriate oligonucleotide primers. The presence of specific PCR products demonstrates that DNA molecules are attached specifically to antigen-antibody complexes, thus indicating the presence of antigen. (Sano *et al., Science* *258*:120-122 (1992)).

For example, Sano *et al.*, *supra*, constructed a streptavidin-protein A chimera that possesses specific binding affinity for biotin and immunoglobulin G. This chimera (i.e., the "linker molecule") was used to attach a biotinylated DNA specifically to antigen-monoclonal antibody complexes that had been immobilized on microtiter plate wells. A segment of the attached DNA was subsequently amplified by PCR.

Detailed Description of the Invention

This invention is directed to neural thread proteins (NTP), genetic sequences coding for an NTP mRNA or antisense mRNA, expression vectors containing the genetic sequences, recombinant hosts transformed therewith, and NTP and antisense RNA produced by such transformed recombinant host expression. This invention further relates to NTP ribozymes, and recombinant DNA molecules which code for NTP ribozymes and NTP antisense oligonucleotides. This invention further relates to antibodies directed against an NTP, as well as the use of NTP antibodies and NTP nucleic acid sequences for detection of the presence of an NTP in biological samples. The invention further relates to the use of NTP coding sequences in gene therapy.

I. Isolation of DNA Sequences Coding for Neuronal Thread Proteins

DNA sequences coding for an NTP are derived from a variety of sources. These sources include genomic DNA, cDNA, synthetic DNA, and combinations thereof.

Human NTP genomic DNA can be extracted and purified from any human cell or tissue, by means well known in the art (for example, *see* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989). The NTP genomic DNA of the invention may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with the 5' promoter region of

the NTP gene sequences and/or with the 3' translational termination region. Further, such genomic DNA may be obtained in association with DNA sequences which encode the 5' nontranslated region of the NTP mRNA and/or with the genetic sequences which encode the 3' nontranslated region. To the extent that a host cell can recognize the transcriptional and/or translational regulatory signals associated with the expression of the mRNA and protein, then the 5' and/or 3' nontranscribed regions of the native gene, and/or, the 5' and/or 3' nontranslated regions of the mRNA, may be retained and employed for transcriptional and translational regulation.

Alternatively, an NTP mRNA can be isolated from any cell which expresses an NTP, and used to produce cDNA by means well known in the art (for example, *see* Sambrook *et al., supra*). Preferably, the mRNA preparation used will be enriched in mRNA coding for an NTP, either naturally, by isolation from cells which produce large amounts of an NTP, or *in vitro*, by techniques commonly used to enrich mRNA preparations for specific sequences, such as sucrose gradient centrifugation, or both. An NTP mRNA may be obtained from mammalian neuronal tissue, or from cell lines derived therefrom. Preferably, human cDNA libraries are constructed from 17-18 week old fetal brain, 2 year old temporal lobe neocortex, end-stage AD cerebral cortex, or from cell lines derived from human neuronal tissue. Such cell lines may include, but are not limited to, central nervous system primitive neuroectodermal tumor cells (such as PNET1 or PNET2, as described herein), neuroblastoma cells (such as SH-Sy5y, as described herein), or human glioma cells (such as A172; ATCC CRL 1620). Alternatively, a rat cDNA library can be prepared from mRNA isolated from rat glioma cells, for example, C6 rat glioma cells (ATCC CCL107).

For cloning into a vector, suitable DNA preparations (either genomic or cDNA) are randomly sheared or enzymatically cleaved, respectively, and ligated into appropriate vectors to form a recombinant gene (either genomic or cDNA) library. A DNA sequence encoding an NTP may be inserted into a vector in accordance with conventional techniques, including blunt-ending

or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulation are disclosed by Sambrook *et al., supra*, and are well known in the art.

Libraries containing NTP clones may be screened and the NTP clones identified by any means which specifically selects for NTP DNA such as, for example: 1) by hybridization with an appropriate nucleic acid probe(s) containing a sequence specific for the DNA of this protein; or, 2) by hybridization-selected translational analysis in which native mRNA hybridizes to the clone in question, is translated *in vitro*, and the translation products are further characterized; or, 3) if the cloned DNA sequences are themselves capable of expressing mRNA, by immunoprecipitation of a translated NTP product produced by the host containing the clone.

Oligonucleotide probes specific for an NTP which can be used to identify clones to this protein can be designed from knowledge of the amino acid sequence of the corresponding NTP, or homologous regions of the PTP. Alternatively, oligonucleotide probes can be designed from knowledge of the nucleotide sequence of PTP (de la Monte *et al., J. Clin. Invest. 86:*1004-1013 (1990)).

The suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the NTP gene (or which is complementary to such an oligonucleotide, or set of oligonucleotides) may be synthesized by means well known in the art (for example, *see* Sambrook *et al., supra*). Techniques of nucleic acid hybridization and clone identification are disclosed by Sambrook *et al., supra*. Those members of the above-described gene library which are found to be capable of such hybridization are then analyzed to determine the extent and nature of the NTP encoding sequences which they contain.

To facilitate the detection of the desired NTP coding sequence, the above-described DNA probe is labeled with a detectable group. Such

detectable group can be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of nucleic acid hybridization and in general most any label useful in such methods can be applied to the present invention. Particularly useful are radioactive labels including ^{32}P, ^{3}H, ^{14}C, ^{125}I, or the like. Any radioactive label may be employed which provides for an adequate signal and has sufficient half-life. The DNA probe may be labeled, for example, by nick-translation, by T4 DNA polymerase replacement synthesis, or by random priming, among other methods well known in the art (*see* Sambrook *et al. supra*).

Alternatively, DNA probes can be labeled with non-radioactive markers such as biotin, an enzyme, or fluorescent group.

In an alternative method of cloning NTP DNA sequences, NTP cDNAs are obtained by direct cloning of cDNAs from cell lines and brain tissue, using the 3'- and 5'-RACE methods, as described herein. Preferably, a human neuroectodermal tumor cell line or AD brain tissue is used as a source of mRNA.

II. Expressing the Gene Coding for NTP

The above-discussed methods are, therefore, capable of identifying DNA sequences which are code for an NTP or fragments thereof. In order to further characterize such DNA sequences, and in order to produce the recombinant protein, it is desirable to express the proteins which the DNA sequences encode.

To express an NTP, transcriptional and translational signals recognizable by an appropriate host are necessary. The cloned NTP DNA sequences, obtained through the methods described above, and preferably in double-stranded form, may be "operably linked" to sequences controlling transcriptional expression in an expression vector, and introduced into a host cell, either prokaryotic or eukaryotic, to produce recombinant NTP. Depending upon which strand of the NTP coding sequence is operably linked

to the sequences controlling transcriptional expression, it is also possible to express an NTP antisense RNA.

Expression of the NTP in different hosts may result in different post-translational modifications which may alter the properties of the NTP. Preferably, the present invention encompasses the expression of an NTP in eukaryotic cells, and especially mammalian, insect, and yeast cells. Especially preferred eukaryotic hosts are mammalian cells. Mammalian cells provide post-translational modifications to recombinant NTP which include folding and/or phosphorylation. Most preferably, mammalian host cells include human CNS primitive neuroectodermal tumor cells, human neuroblastoma cells, human glioma cells, or rat glioma cells. Especially preferred primitive neuroectodermal tumor cells include PNET1 and PNET2, especially preferred human glioblastoma cells include Hg16 and Hg17, especially preferred human glioma cells include A172, and especially preferred rat glioma cells include C6 (*see* Example 1).

Alternatively, an NTP may be expressed by prokaryotic host cells. Preferably, a recombinant NTP is expressed by such cells as a fusion protein, as described herein. An especially preferred prokaryotic host is *E. coli*. Preferred strains of *E. coli* include Y1088, Y1089, CSH18, ER1451, and ER1647 (*see*, for example, *Molecular Biology LabFax*, Brown, T.A., Ed., Academic Press, New York (1991)). An alternative preferred host is *Bacillus subtilus*, including such strains as BR151, YB886, MI119, MI120, and B170 (*see*, for example, Hardy, "Bacillus Cloning Methods," in *DNA Cloning: A Practical Approach*, IRL Press, Washington, D.C. (1985)).

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which in turn contain transcriptional regulatory information and such sequences are "operably linked" to the nucleotide sequence which encodes the protein.

Two sequences of a nucleic acid molecule are said to be operably linked when they are linked to each other in a manner which either permits both sequences to be transcribed onto the same RNA transcript, or permits an

RNA transcript, begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and any other "second" sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked second sequence. In order to be operably linked it is not necessary that two sequences be immediately adjacent to one another.

The promoter sequences of the present invention may be either prokaryotic, eukaryotic or viral. Suitable promoters are repressible, constitutive, or inducible. Examples of suitable prokaryotic promoters include promoters capable of recognizing the T4 polymerases (Malik *et al.*, *J. Biol. Chem.* *263*:1174-1181 (1984); Rosenberg *et al.*, *Gene 59*:191-200 (1987); Shinedling *et al.*, *J. Molec. Biol. 195*:471-480 (1987); Hu *et al.*, *Gene 42*:21-30 (1986)), T3, Sp6, and T7 (Chamberlin *et al.*, *Nature 228*:227-231 (1970); Bailey *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 80*:2814-2818 (1983); Davanloo *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 81*:2035-2039 (1984)); the P_R and P_L promoters of bacteriophage lambda (*The Bacteriophage Lambda*, Hershey, A.D., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1973); *Lambda II*, Hendrix, R.W., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1980)); the trp, recA, heat shock, and lacZ promoters of *E. coli*; the α-amylase (Ulmanen *et al.*, *J. Bacteriol. 162*:176-182 (1985)) and the delta-28-specific promoters of *B. subtilis* (Gilman *et al.*, *Gene 32*:11-20 (1984)); the promoters of the bacteriophages of *Bacillus* (Gryczan, In: *The Molecular Biology of the Bacilli*, Academic Press, Inc., NY (1982)); *Streptomyces* promoters (Ward *et al.*, *Mol. Gen. Genet. 203*:468-478 (1986)); the *int* promoter of bacteriophage lambda; the *bla* promoter of the ß-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325, etc. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol. 1*:277-282 (1987); Cenatiempo, *Biochimie 68*:505-516 (1986); Watson *et al.*, In: *Molecular Biology of the Gene*, Fourth Edition, Benjamin Cummins, Menlo Park, CA (1987); Gottesman, *Ann. Rev. Genet. 18*:415-442 (1984); and Sambrook *et al.*, *supra*.

Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer *et al., J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, *et al., Nature (London) 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, *et al., Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, *et al., Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)). All of the above listed references are incorporated by reference herein.

Strong promoters are the most preferred promoters of the present invention. Examples of such preferred promoters are those which recognize the T3, SP6 and T7 polymerase promoters; the P_L promoter of bacteriophage lambda; the *recA* promoter and the promoter of the mouse metallothionein I gene. The most preferred promoter for expression in prokaryotic cells is one which is capable of recognizing the T7 polymerase promoter. The sequences of such polymerase recognition sequences are disclosed by Watson, *et al.* (In: *Molecular Biology of the Gene*, Fourth Edition, Benjamin Cummins, Menlo Park, CA, (1987)). The most preferred promoter for expression in mammalian cells is SV40 (Gorman, "High Efficiency Gene Transfer into Mammalian cells," in *DNA Cloning: A Practical Approach*, Volume II, IRL Press, Washington, D.C., pp. 143-190 (1985)).

III. Methods of Detecting NTP

This invention is directed towards methods of detecting neurological disease in a human subject, utilizing the nucleic acid probes hybridizable to NTP genes or transcripts, or antibodies specific for an NTP. By "neurological disease" is meant Alzheimer's Disease (AD), or other neurodegenerative disorders with the Alzheimer's type pathogenic changes (for example, Parkinson's disease with AD-type neurodegeneration), as well as neuroectodermal tumors, malignant astrocytomas, and glioblastomas. By "human subject" is meant any human being or any developmental form thereof, such as a human embryo or fetus, prior to birth. The diagnostic methods of the present invention do not require invasive removal of neural tissue.

The present invention additionally pertains to assays, both nucleic acid hybridization assays and immunoassays, for detecting the presence of NTP in cells or in the biological fluids of a human subject using light or electron microscopic histology, imaging, radioactive or enzyme based assays, and the like.

a. Nucleic Acid Hybridization Assays

In testing a tissue sample for an NTP using a nucleic acid hybridization assay, RNA can be isolated from tissue by sectioning on a cryostat and lysing the sections with a detergent such as SDS and a chelating agent such as EDTA, optionally with overnight digestion with proteinase K (50 μg/ml). Such tissue is obtained by autopsy and biopsy. A preferred quantity of tissue is in the range of 1-10 milligrams. Protein is removed by phenol and chloroform extractions, and nucleic acids are precipitated with ethanol. RNA is isolated by chromatography on an oligo dT column and then eluted therefrom. Further fractionation can also be carried out, according to methods well known to those of ordinary skill in the art.

A number of techniques for molecular hybridization are used for the detection of DNA or RNA sequences in tissues; each has certain advantages and disadvantages. When large amounts of tissue are available, analysis of hybridization kinetics provides the opportunity to accurately quantitate the amount of DNA or RNA present, as well as to distinguish sequences that are closely related but not identical to the probe, and determine the percent homology.

Reactions are run under conditions of hybridization (Tm-25°C) in which the rate of reassociation of the probe is optimal (Wetmur *et al., J. Mol. Biol. 31*:349-370 (1968)). The kinetics of the reaction are second- order when the sequences in the tissue are identical to those of the probe; however, the reaction exhibits complex kinetics when probe sequences have partial homology to those in the tissue (Sharp *et al., J. Mol. Biol. 86*:709-726 (1974)).

The ratio of probe to cell RNA is determined by the sensitivity desired. To detect one transcript per cell would require about 100 pg of probe per μg of total cellular DNA or RNA. The nucleic acids are mixed, denatured, brought to the appropriate salt concentration and temperature, and allowed to hybridize for various periods of time. The rate of reassociation can be determined by quantitating the amount of probe hybridized either by hydroxy apatite chromatography (Britten *et al., Science 161*:529-540 (1968)) or S1 nuclease digestion (Sutton, *Biochim. Biophys. Acta 240*:522-531 (1971)).

A more flexible method of hybridization is the northern blot technique. This technique offers variability in the stringency of the hybridization reaction, as well as determination of the state of the retroviral sequences in the specimen under analysis. Northern analysis can be performed as described herein.

A major consideration associated with hybridization analysis of DNA or RNA sequences is the degree of relatedness the probe has with the sequences present in the specimen under study. This is important with the blotting technique, since a moderate degree of sequence homology under

nonstringent conditions of hybridization can yield a strong signal even though the probe and sequences in the sample represent non-homologous genes.

The particular hybridization technique is not essential to the invention, any technique commonly used in the art being within the scope of the present invention. Typical probe technology is described in United States Patent 4,358,535 to Falkow *et al.*, incorporated by reference herein. For example, hybridization can be carried out in a solution containing 6 x SSC (10 x SSC: 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0), 5 x Denhardt's (1 x Denhardt's: 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.02% Ficoll 400), 10 mM EDTA, 0.5% SDS and about 10^7 cpm of nick-translated DNA for 16 hours at 65°C.

The labeled probes, as described above, provide a general diagnostic method for detection of an NTP in tissue. The method is reasonably rapid, has a simple protocol, has reagents which can be standardized and provided as commercial kits, and allows for rapid screening of large numbers of samples.

In one method for carrying out the procedure, a clinical isolate containing RNA transcripts is fixed to a support. The affixed nucleic acid is contacted with a labeled polynucleotide having a base sequence complementary or homologous to the coding strand of the NTP gene.

The hybridization assays of the present invention are particularly well suited for preparation and commercialization in kit form, the kit comprising a carrier means compartmentalized to receive one or more container means (vial, test tube, etc.) in close confinement, each of said container means comprising one of the separate elements to be used in hybridization assay.

For example, there may be a container means containing NTP cDNA molecules suitable for labeling by "nick translation" (*see*, for example, *Sambrook et al., supra,* for standard methodology), or labeled NTP cDNA or RNA molecules. Further container means may contain standard solutions for nick translation of NTP cDNA comprising DNA polymerase I/DNase I and unlabeled deoxyribonucleotides (i.e., dCTP, dTTP, dGTP, and dATP).

The presence of NTP RNA is determined by the variation in the appearance and/or quantity of probe-related RNA in tested tissue.

The DNA probes of this invention can also be used for differential diagnosis of hereditary or familial AD and non-hereditary or sporadic AD. The familial form of AD often occurs at an earlier age and is associated with Down's syndrome in the family. Thus, a genetic test for familial AD allows for genetic counseling of families. While much effort has been directed toward characterizing a genetic marker for familial AD (Gusella, *FASEB J 3*:2036-2041 (1989); Hooper, *J NIH Res. 4*:48-54 (1992)), genetic linkage analysis only identifies a genetic marker sequence without providing the knowledge of the function of the genomic sequence. In contrast, the cDNA probes described herein and obtained from individuals with sporatic AD encode a known protein of known function which is over-expressed in brain tissue of patients with AD.

Most cases of the AD disorder appear to be the sporadic form, although there are well-documented familial cases (Gusella, *supra*; *Harrison's Principles of Internal Medicine*, Braunwald *et al.*, Eds., Eleventh Edition, Mc-Graw-Hill Book Company, New York, pp. 2012-2013 (1987)). A patient with familial AD, unlike a patient with sporadic AD, inherited the predisposing mutation through the germ cells. Some of the familial cases have been shown to follow an autosomal dominant pattern of inheritance (*Id.*). Thus, the DNA of a patient with familial AD will contain the inherited genetic alteration which is absent from the DNA of a patient with sporadic AD.

A method of differentiating between sporadic and familial AD in a human subject involves obtaining a biological sample from the human subject who is suspected of having Alzheimer's Disease. Then, DNA is purified from the biological sample. Finally, the DNA is contacted with a NTP DNA probe under conditions of hybridization. Familial AD is indicated by the detection of a hybrid of the probe and the DNA, whereas sporadic AD is indicated by the absence of detection of hybridization.

For example, the biological sample can be a blood sample which is subjected to differential centrifugation to enrich for white blood cells within three days of collection (Park, "PCR in the Diagnosis of Retinoblastoma," in *PCR Protocols*, Innis *et al.*, Eds., Academic Press, Inc., New York, pp. 407-415 (1990)). The DNA sample can be prepared using the sodium N-lauroylsarcosine-Proteinase K, phenol, and RNase method (Sambrook *et al.*, *supra*). DNA analysis can be performed by digesting the DNA sample, preferably 5 micrograms, with a restriction endonuclease (such as *Hind*III). Digested DNA is then fractionated using agarose gel electrophoresis, preferably, a 1% horizontal agarose gel, for 18 hours in a buffer preferably containing 89 mM Tris-Hcl (pH 8), 89 mM sodium borate and 2 mM EDTA (Gusella *et al., Nature 306*:234-238 (1983)). Southern analysis can be performed using conventional techniques (Sambrook *et al., supra*), and the labelled AD cDNA probes can be hybridized under conditions described above. The preferred DNA probes for this differential diagnosis method include 1-9a, AD3-4, AD4-4 and G2-2 PstI.

b. Immunoassays

Antibodies directed against an NTP can be used, as taught by the present invention, to detect and diagnose AD. Various histological staining methods, including immunohistochemical staining methods, may also be used effectively according to the teaching of the invention. Silver stain is but one method of visualizing NTP. Other staining methods useful in the present invention will be obvious to the artisan, the determination of which would not involve undue experimentation (*see generally*, for example, *A Textbook of Histology*, Eds. Bloom and Fawcett, W.B. Saunders Co., Philadelphia (1964)).

One screening method for determining whether a given compound is an NTP functional derivative comprises, for example, immunoassays employing radioimmunoassay (RIA) or enzyme-linked immunosorbant assay (ELISA) methodologies, based on the production of specific antibodies

(monoclonal or polyclonal) to an NTP. For these assays, biological samples are obtained by venepuncture (blood), spinal tap (cerebral spinal fluid (CSF)), urine and other body secretions such as sweat and tears. For example, in one form of RIA, the substance under test is mixed with diluted antiserum in the presence of radiolabeled antigen. In this method, the concentration of the test substance will be inversely proportional to the amount of labeled antigen bound to the specific antibody and directly related to the amount of free labeled antigen. Other suitable screening methods will be readily apparent to those of skill in the art.

The present invention also relates to methods of detecting an NTP or functional derivatives in a sample or subject. For example, antibodies specific for an NTP, or a functional derivative, may be detectably labeled with any appropriate marker, for example, a radioisotope, an enzyme, a fluorescent label, a paramagnetic label, or a free radical.

Alternatively, antibodies specific for an NTP, or a functional derivative, may be detectably labeled with DNA by the technique of immuno-polymerase chain reaction (Sano *et al., Science 258*: 120-122 (1992)). The polymerase chain reaction (PCR) procedure amplifies specific nucleic acid sequences through a series of manipulations including denaturation, annealing of oligonucleotide primers, and extension of the primers with DNA polymerase (*see*, for example, Mullis *et al.*, U.S. Patent No. 4,683,202; Mullis *et al.*, U.S. Patent No. 4,683,195; Loh *et al., Science 243*:217 (1988)). The steps can be repeated many times, resulting in a large amplification of the number of copies of the original specific sequence. As little as a single copy of a DNA sequence can be amplified to produce hundreds of nanograms of product (Li *et al., Nature 335*:414 (1988)). Other known nucleic acid amplification procedures include transcription-based amplification systems (Kwoh *et al., Proc. Natl. Acad. Sci. USA 86*:1173 (1989); Gingeras *et al.*, WO 88/10315), and the "ligase chain reaction" in which two (or more) oligonucleotides are ligated in the presence of a nucleic acid target having the sequence of the resulting "di-oligonucleotide" thereby amplifying the di-

oligonucleotide (Wu *et al., Genomics* 4:560 (1989); Backman *et al.*, EP 320,308; Wallace, EP 336,731; Orgel, WO 89/09835). For example, the immuno-PCR assay can be carried out by immobilizing various amounts of the test material on the surface of microtiter wells (*see* Sanzo *et al., supra*, page 122, footnote 7). The wells are subsequently incubated with an NTP monoclonal antibody, washed, and then incubated with biotinylated NTP DNA molecules which have been conjugated to streptavidin-protein chimera (*Id.*). This chimera binds biotin (via the streptavidin moiety) and the Fc portion of an immunoglobulin G molecule (via the protein A moiety) (*Id.*, at 120; Sanzo *et al.*, *Bio/Technology* 9:1378 (1991)). The wells are then washed to remove unbound conjugates. Any NTP present in the test material will be bound by the NTP monoclonal antibody, which in turn, is bound by the protein A moiety of the biotinylated NTP DNA - streptavidin-protein A conjugate. Then, the NTP DNA sequences are amplified using PCR. Briefly, the microtiter wells are incubated with deoxyribonucleoside triphosphates, NTP oligonucleotide primers, and Taq DNA polymerase (*see* Sanzo *et al., supra*, page 122, footnote 11). An automated thermal cycler (such as the PTC-100-96 Thermal Cycler, MJ Research, Inc.) can be used to perform PCR under standard conditions (*Id.*). The PCR products are then analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Methods of making and detecting such detectably labeled antibodies or their functional derivatives are well known to those of ordinary skill in the art, and are described in more detail below. Standard reference works setting forth the general principles of immunology include the work of Klein (*Immunology: The Science of Self-Nonself Discrimination*, John Wiley & Sons, New York (1982)); Kennett *et al.* (*Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, New York (1980)); Campbell ("Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13 (Burdon, R., *et al.*, eds.). Elsevier, Amsterdam (1984)); and Eisen (In: *Microbiology*, 3rd Ed. (Davis, *et al.*, Harper & Row, Philadelphia (1980)).

The term "antibody" refers both to monoclonal antibodies which are a substantially homogeneous population and to polyclonal antibodies which are heterogeneous populations. Polyclonal antibodies are derived from the sera of animals immunized with an antigen. Monoclonal antibodies (mAbs) to specific antigens may be obtained by methods known to those skilled in the art. *See*, for example, Kohler and Milstein, *Nature 256*:495-497 (1975) and U.S. Patent No. 4,376,110. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

The monoclonal antibodies, particularly mAbs Th7, Th9, and Th10 used in the present invention, may be prepared as previously described (Gross *et al., J. Clin. Invest. 76*:2115-2126 (1985); Ozturk *et al., Proc. Natl. Acad. Sci. USA 86*:419-423 (1989); de la Monte *et. al., J. Clin. Invest. 86*:1004-1013 (1990); de la Monte *et. al., J. Neurol. Sci. 113*:152-164 (1992); de la Monte *et al., Ann. Neurol. 32*:733-742 (1992)). The Th monoclonal antibodies were generated against the purified pancreatic form of thread protein (*Id.*). NTP-specific polyclonal and monoclonal antibodies can also be generated against a substantially pure NTP isolated from recombinant hosts (for example, *see* Carroll *et al.*, "Production and Purification of Polyclonal Antibodies to the Foreign Segment of ß-Galactosidase Fusion Proteins," in *DNA Cloning: A Practical Approach*, Volume III, IRL Press, Washington, D.C., pp. 89-111 (1987); Mole *et al.*, "Production of Monoclonal Antibodies Against Fusion Proteins Produced in *Escherichia coli*," in *DNA Cloning: A Practical Approach*, Volume III, IRL Press, Washington, D.C., pp. 113-1139 (1987)). Alternatively, NTP-specific polyclonal and monoclonal antibodies can be generated against a substantially pure NTP isolated from biological material such as brain tissue and cell lines, by using well known techniques.

For example, monoclonal antibodies specific for the various NTP molecules of approximately, 8, 14, 17, 21, 26 kDa and 42 kDa molecular weights may be prepared from recombinant-derived proteins, which are expressed, isolated and purified from the cDNA (i.e., 1-9a), genomic clones (G2-2 *PstI*) and AD-NTP 3-4 cDNA clones. These NTP molecules are

derived from the above cDNA's and genomic clones, inserted and produced in suitable expression vectors (*see* Figures 2A and 2B). Since there are regions of 60-70% homology in the 5' ends of the 1-9a NTP cDNA and PTP, one can obtain monoclonal antibodies that bind specifically to the NTP recombinant proteins and not to the pancreatic form by performing routine differential screening (*see*, for example, de la Monte *et al.*, *J. Clin. Invest. 86*: 1004-1013 (1990)). Although there will be monoclonal antibodies that bind to both NTP and PTP, it will be possible to generate NTP-specific monoclonal antibodies because there is a substantial sequence divergence between NTP molecules of various forms (e.g., 8, 14, 17, 21, 26 and 42 kDa) and because an epitope may be defined by as few as 6-8 amino acids.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl *et al.*, *J. Nucl. Med. 24*:316-325 (1983)).

It will be appreciated that Fab and $F(ab')_2$ and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of an NTP according to the methods disclosed herein in order to detect and diagnose AD in the same manner as an intact antibody. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one, or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies, or fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the presence of cells which contain the NTP antigens. Thus, the antibodies (or fragments thereof) useful in the present invention may be employed histologically to detect or visualize the presence of an NTP.

Such an assay for an NTP typically comprises incubating a biological sample from said subject suspected of having such a condition in the presence of a detectably labeled binding molecule (e.g., antibody) capable of identifying an NTP, and detecting said binding molecule which is bound in a sample.

Thus, in this aspect of the invention, a biological sample may be treated with nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled NTP-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support may then be detected by conventional means.

By "solid phase support" is intended any support capable of binding antigen or antibodies. Well-known supports, or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical,

as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

One embodiment for carrying out the diagnostic assay of the present invention on a biological sample containing an NTP, comprises:

(a) contacting a detectably labeled NTP-specific antibody with a solid support to effect immobilization of said NTP-specific antibody or a fragment thereof;

(b) contacting a sample suspected of containing an NTP with said solid support;

(c) incubating said detectably labeled NTP-specific antibody with said support for a time sufficient to allow the immobilized NTP-specific antibody to bind to the NTP;

(d) separating the solid phase support from the incubation mixture obtained in step (c); and

(e) detecting the bound label and thereby detecting and quantifying NTP.

Alternatively, labeled NTP-specific antibody/NTP complexes in a sample may be separated from a reaction mixture by contacting the complex with an immobilized antibody or protein which is specific for an immunoglobulin, e.g., *Staphylococcus* protein A, *Staphylococcus* protein G, anti-IgM or anti-IgG antibodies. Such anti-immunoglobulin antibodies may be polyclonal, but are preferably monoclonal. The solid support may then be washed with a suitable buffer to give an immobilized NTP/labeled NTP-specific antibody complex. The label may then be detected to give a measure of an NTP.

This aspect of the invention relates to a method for detecting an NTP or a fragment thereof in a sample comprising:

(a) contacting a sample suspected of containing an NTP with an NTP-specific antibody or fragment thereof which binds to NTP; and

(b) detecting whether a complex is formed.

The invention also relates to a method of detecting an NTP in a sample, further comprising:

(c) contacting the mixture obtained in step (a) with an Fc binding molecule, such as an antibody, *Staphylococcus* protein A, or *Staphylococcus* protein G, which is immobilized on a solid phase support and is specific for the NTP-specific antibody to give a NTP/NTP-specific antibody immobilized antibody complex;

(d) washing the solid phase support obtained in step (c) to remove unbound NTP/NTP-specific antibody complex;

(e) and detecting the label bound to said solid support.

Of course, the specific concentrations of detectably labeled antibody and NTP, the temperature and time of incubation, as well as other assay conditions may be varied, depending on various factors including the concentration of an NTP in the sample, the nature of the sample, and the like. The binding activity of a given lot of anti-NTP antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which the NTP-specific antibody can be detectably labeled is by linking the same to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be

used to detectably label the NTP-specific antibody include, but are not limited to, malate dehydrogenase. staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, ß-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

Detection may be accomplished using any of a variety of immunoassays. For example, by radioactively labeling the NTP-specific antibodies or antibody fragments, it is possible to detect NTP through the use of radioimmune assays. A good description of a radioimmune assay may be found in *Laboratory Techniques and Biochemistry in Molecular Biology*, by Work, *et al.*, North Holland Publishing Company, NY (1978), with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, incorporated by reference herein.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and preferably ^{125}I.

It is also possible to label the NTP-specific antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine.

The NTP-specific antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the NTP-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The NTP-specific antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-

tagged NTP-specific antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

The NTP-specific antibody may also be labeled with biotin and then reacted with avidin. A biotin-labeled DNA fragment will be linked to the NTP-biotinylated monoclonal antibody by an avidin bridge. NTP molecules are then detected by polymerase chain reaction (PCR) amplification of the DNA fragment with specific primers (Sano *et al.*, *Science 258*: 120-122 (1992)).

Likewise, a bioluminescent compound may be used to label the NTP-specific antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the NTP-specific antibody may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

The detection of foci of such detectably labeled antibodies is indicative of a disease or dysfunctional state as previously described. For the purposes of the present invention, the NTP which is detected by this assay may be present in a biological sample. Any sample containing an NTP can be used. However, one of the benefits of the present diagnostic invention is that invasive tissue removal may be avoided. Therefore, preferably, the sample

is a biological solution such as, for example, cerebrospinal fluid, amniotic fluid, blood, serum, urine and the like. However, the invention is not limited to assays using only these samples, it being possible for one of ordinary skill in the art to determine suitable conditions which allow the use of other samples.

For example, the three-site monoclonal antibody-based immunoradiometric assays (M-IRMA) may be used to measure NTP levels in a biological fluid, such as CSF. It is possible to obtain, by spinal tap, on a routine basis, CSF from individuals suspected of having AD. Thus, the diagnosis of AD can be established by a simple, non-invasive immunoassay which reveals NTP levels greatly increased over normal levels.

In one embodiment, as described above, this examination for AD is accomplished by removing samples of biological fluid and incubating such samples in the presence of detectably labeled antibodies (or antibody fragments). In a preferred embodiment, this technique is accomplished in a non-invasive manner through the use of magnetic imaging, fluorography, etc.

Preferably, the detection of cells which express an NTP may be accomplished by *in vivo* imaging techniques, in which the labeled antibodies (or fragments thereof) are provided to a subject, and the presence of the NTP is detected without the prior removal of any tissue sample. Such *in vivo* detection procedures have the advantage of being less invasive than other detection methods, and are, moreover, capable of detecting the presence of NTP in tissue which cannot be easily removed from the patient, such as brain tissue.

Using *in vivo* imaging techniques, it will be possible to differentiate between AD and a brain tumor because NTP will be detected throughout the brain in an AD patient, while NTP will be localized in discrete deposits in the case of brain tumors. For example, in brains of AD patients, NTP will be found in the temporal, parietal and frontal cortices as well as the amygdala and hippocampus. Favored cites for astrocytomas include the cerebrum, cerebellum, thalamus, optic chiasma, and pons (*Harrison's Principles of*

Internal Medicine, Petersdorf *et al.*, Eds., Tenth Edition, McGraw-Hill Book Company, New York, p.2076 (1983)), and glioblastoma multiforme is predominantly cerebral in location (*Id.* at 2075).

There are many different *in vivo* labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioactive isotopes and paramagnetic isotopes. Those of ordinary skill in the art will know of other suitable labels for binding to the antibodies used in the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the antibodies can be done using standard techniques common to those of ordinary skill in the art.

An important factor in selecting a radionuclide for *in vivo* diagnosis is that the half-life of a radionuclide be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation upon the host is minimized. Ideally, a radionuclide used for *in vivo* imaging will lack a particulate emission, but produce a large number of photons in the 140-200 keV range, which maybe readily detected by conventional gamma cameras.

For *in vivo* diagnosis radionuclides may be bound to antibody either directly or indirectly by using an intermediary functional group. Intermediary functional groups which are often used in binding radioisotopes which exist as metallic ions to immunoglobulins are DTPA and EDTA. Typical examples of ions which can be bound to immunoglobulins are ^{99m}Tc, ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, ^{125}I, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

For diagnostic *in vivo* imaging, the type of detection instrument available is a major factor in selecting a given radionuclide. The radionuclide chosen must have a type of decay which is detectable for a given type of instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention. For example, PET, gamma, beta, and MRI detectors can be used to visualize diagnostic imagining.

The antibodies useful in the invention can also be labeled with paramagnetic isotopes for purposes of *in vivo* diagnosis. Elements which are particularly useful, as in Magnetic Resonance Imaging (MRI), include ^{157}Gd, ^{55}Mn, ^{162}Dy, and ^{56}Fe.

The antibodies (or fragments thereof) useful in the present invention are also particularly suited for use in *in vitro* immunoassays to detect the presence of an NTP in body tissue, fluids (such as CSF), or cellular extracts. In such immunoassays, the antibodies (or antibody fragments) may be utilized in liquid phase or, preferably, bound to a solid-phase carrier, as described above.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, *et al.* (*Clin. Chim. Acta* *70*:1-31 (1976)) and Schurs, *et al.* (*Clin. Chim. Acta* *81*:1-40 (1977)). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method, all of which methods are incorporated by reference herein.

In situ detection may be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of an NTP, but also the distribution of an NTP on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

The binding molecules of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or

"sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested (i.e., CSF) and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and then contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay may be a simple "yes/no" assay to determine whether antigen is present or may be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of antigen. Such "two-site" or "sandwich" assays are described by Wide at pages 199-206 of *Radioimmune Assay Method*, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays.

The above-described *in vitro* or *in vivo* detection methods may be used in the detection and diagnosis of AD without the necessity of removing tissue. Such detection methods may be used to assist in the determination of the stage of neurological deterioration in AD by evaluating and comparing the concentration of an NTP in the biological sample.

As used herein, an effective amount of a diagnostic reagent (such as an antibody or antibody fragment) is one capable of achieving the desired diagnostic discrimination and will vary depending on such factors as age, condition, sex, the extent of disease of the subject, counterindications, if any, and other variables to be adjusted by the physician. The amount of such materials which are typically used in a diagnostic test are generally between 0.1 to 5 mg, and preferably between 0.1 to 0.5 mg.

The assay of the present invention is also ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of said container means comprising the separate elements of the immunoassay.

For example, there may be a container means containing a first antibody immobilized on a solid phase support, and a further container means containing a second detectably labeled antibody in solution. Further container means may contain standard solutions comprising serial dilutions of the NTP to be detected. The standard solutions of an NTP may be used to prepare a standard curve with the concentration of NTP plotted on the abscissa and the

detection signal on the ordinate. The results obtained from a sample containing an NTP may be interpolated from such a plot to give the concentration of the NTP.

IV. Isolation of NTP

The NTP proteins or fragments of this invention may be obtained by expression from recombinant DNA as described above. Alternatively, an NTP may be purified from biological material.

For purposes of the present invention, one method of purification which is illustrative, without being limiting, consists of the following steps.

A first step in the purification of an NTP includes extraction of the NTP fraction from a biological sample, such as brain tissue or CSF, in buffers, with or without solubilizing agents such as urea, formic acid, detergent, or thiocyanate.

A second step includes subjecting the solubilized material to ion-exchange chromatography on Mono-Q or Mono-S columns (Pharmacia LKB Biotechnology, Inc; Piscataway, NJ). Similarly, the solubilized material may be separated by any other process wherein molecules can be separated according to charge density, charge distribution and molecular size, for example. Elution of the NTP from the ion-exchange resin are monitored by an immunoassay, such as M-IRMA, on each fraction. Immunoreactive peaks would are then dialyzed, lyophilized, and subjected to molecular sieve, or gel chromatography.

Molecular sieve or gel chromatography is a type of partition chromatography in which separation is based on molecular size. Dextran, polyacrylamide, and agarose gels are commonly used for this type of separation. One useful gel for the present invention is Sepharose 12 (Pharmacia LKB Biotechnology, Inc.). However, other methods, known to those of skill in the art may be used to effectively separate molecules based on size.

A fourth step in a purification protocol for an NTP includes analyzing the immunoreactive peaks by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a further gel chromatographic purification step, and staining, such as, for example, silver staining.

A fifth step in a purification method includes subjecting the NTP obtained after SDS-PAGE to affinity chromatography, or any other procedure based upon affinity between a substance to be isolated and a molecule to which it can specifically bind. For further purification of an NTP, affinity chromatography on Sepharose conjugated to anti-NTP mAbs (such as Th9, or specific mABs generated against substantially pure NTP) can be used. Alternative methods, such as reverse-phase HPLC, or any other method characterized by rapid separation with good peak resolution are useful.

Another method to purify an NTP is to use concentrated CSF obtained from patients with AD. For this procedure, 30-40 milliliters are concentrated by lyophilization or Amicon filtration or the like, and subjected to two dimensional gel electrophoresis. Proteins are separated in one direction by charge in a pH gradient and then, subjected to molecular sieve chromatography in the other direction by polyacrylamide gel electrophoresis. NTP-immunoreactive proteins are identified as spots by the Th monoclonal antibodies (for example, Th 9) using Western blot anaylsis. The gel is cut and NTP proteins are eluted from the gel. NTP purified in this manner can be sequenced or used to make new monoclonal antibodies.

It will be appreciated that other purification steps may be substituted for the preferred method described above. Those of skill in the art will be able to devise alternate purification schemes without undue experimentation.

V. Gene Therapy Using Antisense Oligonucleotides and Ribozymes

Antisense oligonucleotides have been described as naturally occurring biological inhibitors of gene expression in both prokaryotes (Mizuno *et al.*, *Proc. Natl. Acad. Sci. USA 81*:1966-1970 (1984)) and eukaryotes (Heywood,

Nucleic Acids Res. 14:6771-6772 (1986)), and these sequences presumably function by hybridizing to complementary mRNA sequences, resulting in hybridization arrest of translation (Paterson, *et al.*, *Proc. Natl. Acad. Sci. USA, 74*:4370-4374 (1987)).

Antisense oligonucleotides are short synthetic DNA or RNA nucleotide molecules formulated to be complementary to a specific gene or RNA message. Through the binding of these oligomers to a target DNA or mRNA sequence, transcription or translation of the gene can be selectively blocked and the disease process generated by that gene can be halted (*see*, for example, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press (1989)). The cytoplasmic location of mRNA provides a target considered to be readily accessible to antisense oligodeoxynucleotides entering the cell; hence much of the work in the field has focused on RNA as a target. Currently, the use of antisense oligodeoxynucleotides provides a useful tool for exploring regulation of gene expression *in vitro* and in tissue culture (Rothenberg, *et al.*, *J. Natl. Cancer Inst. 81*:1539-1544 (1989)).

Antisense therapy is the administration of exogenous oligonucleotides which bind to a target polynucleotide located within the cells. For example, antisense oligonucleotides may be administered systemically for anticancer therapy (Smith, International Application Publication No. WO 90/09180). As described herein, NTP-related proteins are produced by neuroectodermal tumor cells, malignant astrocytoma cells, glioblastoma cells, and in relatively high concentrations (i.e, relative to controls) in brain tissue of AD patients. Thus, NTP antisense oligonucleotides of the present invention may be active in treatment against AD, as well as neuroectodermal tumors, malignant astrocytomas, and glioblastomas.

The NTP antisense oligonucleotides of the present invention include derivatives such as S-oligonucleotides (phosphorothioate derivatives or S-oligos, *see*, Jack Cohen, *supra*). S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligo) in which a nonbridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-

oligos of the present invention may be prepared by treatment of the corresponding O-oligos with *3H*-1,2-benzodithiol-3-one-1,1-dioxide which is a sulfur transfer reagent. *See* Iyer *et al., J. Org. Chem. 55*:4693-4698 (1990); and Iyer *et al., J. Am. Chem. Soc. 112*:1253-1254 (1990), the disclosures of which are fully incorporated by reference herein.

As described herein, sequence anaylsis of an NTP cDNA clone shows that NTP contains sequences which are nonhomologous to PTP DNA sequences (*see* Figure 9). Thus, the NTP antisense oligonucleotides of the present invention may be RNA or DNA which is complementary to and stably hybridizes with such sequences which are specific for an NTP. Use of an oligonucleotide complementary to this region allows for the selective hybridization to NTP mRNA and not to mRNA specifying PTP. Preferably, the NTP antisense oligonucleotides of the present invention are a 15 to 30-mer fragment of the antisense DNA molecule coding for the nonhomologous sequences of the AD 3-4 cDNA, such as:

1. 5'-CCGATTCCAACAGACCATCAT-3' [SEQ ID NO: 1];
2. 5'-CCAACAGACCATCATTCCACC-3' [SEQ ID NO: 2]; and
3. 5'-CCAAACCGATTCCAACAGACC-3' [SEQ ID NO: 3].

Preferred antisense oligonucleotides bind to the 5'-end of the AD10-7 mRNA. Such antisense oligonucleotides may be used to down regulate or inhibit expression of the NTP gene. Examples of such antisense oligonucleotides (30-mers) include:

1. 5'-CCTGGGCAACAAGAGCGAAAACTCCATCTC-3' [SEQ ID NO: 4];
2. 5'-ATCGCTTGAACCCGGGAGGCGGAGGTTGCG-3' [SEQ ID NO: 5]; and
3. 5'-GGGGAGGCTGAGGCAGGAGAATCGCTTGAA-3'[SEQ ID NO: 6].

Included as well in the present invention are pharmaceutical compositions comprising an effective amount of at least one of the NTP antisense oligonucleotides of the invention in combination with a pharma-

ceutically acceptable carrier. In one embodiment, a single NTP antisense oligonucleotide is utilized. In another embodiment, two NTP antisense oligonucleotides are utilized which are complementary to adjacent regions of the NTP genome. Administration of two NTP antisense oligonucleotides which are complementary to adjacent regions of the genome or corresponding mRNA may allow for more efficient inhibition of NTP genomic transcription or mRNA translation, resulting in more effective inhibition of NTP production.

Preferably, the NTP antisense oligonucleotide is coadministered with an agent which enhances the uptake of the antisense molecule by the cells. For example, the NTP antisense oligonucleotide may be combined with a lipophilic cationic compound which may be in the form of liposomes. The use of liposomes to introduce nucleotides into cells is taught, for example, in U.S. Patent Nos. 4,897,355 and 4,394,448, the disclosures of which are incorporated by reference in their entirety. *See also* U.S. Patent Nos. 4,235,871, 4,231,877, 4,224,179, 4,753,788, 4,673,567, 4;247,411, 4,814,270 for general methods of preparing liposomes comprising biological materials.

Alternatively, the NTP antisense oligonucleotide may be combined with a lipophilic carrier such as any one of a number of sterols including cholesterol, cholate and deoxycholic acid. A preferred sterol is cholesterol.

In addition, the NTP antisense oligonucleotide may be conjugated to a peptide that is ingested by cells. Examples of useful peptides include peptide hormones, antigens or antibodies, and peptide toxins. By choosing a peptide that is selectively taken up by the neoplastic cells, specific delivery of the antisense agent may be effected. The NTP antisense oligonucleotide may be covalently bound via the 5'OH group by formation of an activated aminoalkyl derivative. The peptide of choice may then be covalently attached to the activated NTP antisense oligonucleotide via an amino and sulfhydryl reactive hetero bifunctional reagent. The latter is bound to a cysteine residue present in the peptide. Upon exposure of cells to the NTP antisense oligonucleotide

bound to the peptide, the peptidyl antisense agent is endocytosed and the NTP antisense oligonucleotide binds to the target NTP mRNA to inhibit translation (Haralambid *et al.*, WO 8903849; Lebleu *et al.*, EP 0263740).

The NTP antisense oligonucleotides and the pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intra-peritoneal, or transdermal routes. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

Compositions within the scope of this invention include all compositions wherein the NTP antisense oligonucleotide is contained in an amount effective to achieve inhibition of proliferation and/or stimulate differentiation of the subject cancer cells, or alleviate AD. While individual needs vary, determination of optimal ranges of effective amounts of each component is with the skill of the art. Typically, the NTP antisense oligonucleotide may be administered to mammals, e.g. humans, at a dose of 0.005 to 1 mg/kg/day, or an equivalent amount of the pharmaceutically acceptable salt thereof, per day of the body weight of the mammal being treated.

Alternatively, antisense oligonucleotides can be prepared which are designed to interfere with transcription of the NTP gene by binding transcribed regions of duplex DNA (including introns, exons, or both) and forming triple helices (Froehler *et al.*, WO 91/06626; Toole, WO 92/10590). Preferred oligonucleotides for triple helix formation are oligonucleotides which have inverted polarities for at least two regions of the oligonucleotide (*Id.*). Such oligonucleotides comprise tandem sequences of opposite polarity such as 3'---5'-L-5'---3', or 5'---3'-L-3'---5', wherein L represents a 0-10 base oligonucleotide linkage between oligonucleotides. The inverted polarity form stabilizes single-stranded oligonucleotides to exonuclease degradation (Froehler *et al., supra*). Preferred triple helix-forming oligonucleotides are based upon SEQ ID NOs 1-3:

1. 3'-TACTACCAGACAACCTTAGCC-5'-L-
   5'-CCGATTCCAACAGACCATCAT-3' [SEQ ID NO: 7];
2. 5'-CCGATTCCAACAGACCATCAT-3'-L-
   3'-TACTACCAGACAACCTTAGCC-5' [SEQ ID NO: 8];
3. 3'-CCACCTTACTACCAGACAACC-5'-L-
   5'-CCAACAGACCATCATTCCACC-3' [SEQ ID NO: 9];
4. 5'-CCAACAGACCATCATTCCACC-3'-L-
   3'-CCACCTTACTACCAGACAACC-5' [SEQ ID NO: 10];
5. 3'-CCAGACAACCTTAGCCAAACC-5'-L-
   5'-CCAAACCGATTCCAACAGACC-3' [SEQ ID NO: 11]; and
6. 5'-CCAAACCGATTCCAACAGACC-3'-L-
   3'-CCAGACAACCTTAGCCAAACC-5' [SEQ ID NO: 12].

Thus, triple helix-forming oligonucleotides 1 and 2 are represented as 3'[SEQ ID NO: 1]5'-L-5'[SEQ ID NO: 1]3' and 5'[SEQ ID NO: 1]3'-L-3'[SEQ ID NO: 1]5', respectively. Triple helix-forming oligonucleotides 3 and 4 are represented as 3'[SEQ ID NO: 2]5'-L-5'[SEQ ID NO: 2]3' and 5'[SEQ ID NO: 2]3'-L-3'[SEQ ID NO: 2]5', respectively. Triple helix-forming oligonucleotides 5 and 6 are represented as 3'[SEQ ID NO: 3]5'-L-5'[SEQ ID NO: 3]3' and 5'[SEQ ID NO: 3]3'-L-3'[SEQ ID NO: 3]5', respectively. Of course, similar triple helix-forming oligonucleotide may be prepared with SEQ ID NOs. 4-6, or fragments thereof.

In therapeutic application, the triple helix-forming oligonucleotides can be formulated in pharmaceutical preparations for a variety of modes of administration, including systemic or localized administration, as described above.

The antisense oligonucleotides of the present invention may be prepared according to any of the methods that are well known to those of ordinary skill in the art, as described above.

Ribozymes provide an alternative method to inhibit mRNA function. Ribozymes may be RNA enzymes, self-splicing RNAs, and self-cleaving RNAs (Cech *et al.*, *Journal of Biological Chemistry 267:*17479-17482 (1992)).

It is possible to construct *de novo* ribozymes which have an endonuclease activity directed in *trans* to a certain target sequence. Since these ribozymes can act on various sequences, ribozymes can be designed for virtually any RNA substrate. Thus, ribozymes are very flexible tools for inhibiting the expression of specific genes and provide an alternative to antisense constructs.

A ribozyme against chloramphenicol acetyltransferase mRNA has been successfully constructed (Haseloff *et al.*, *Nature 334*:585-591 (1988); Uhlenbeck *et al.*, *Nature 328*:596-600 (1987)). The ribozyme contains three structural domains: 1) a highly conserved region of nucleotides which flank the cleavage site in the 5' direction; 2) the highly conserved sequences contained in naturally occurring cleavage domains of ribozymes, forming a base-paired stem; and 3) the regions which flank the cleavage site on both sides and ensure the exact arrangement of the ribozyme in relation to the cleavage site and the cohesion of the substrate and enzyme. RNA enzymes constructed according to this model have already proved suitable *in vitro* for the specific cleaving of RNA sequences (Haseloff *et al.*, *supra*).

Alternatively, hairpin ribozymes may be used in which the active site is derived from the minus strand of the satellite RNA of tobacco ring spot virus (Hampel *et al.*, *Biochemistry 28*:4929-4933 (1989)). Recently, a hairpin ribozyme was designed which cleaves human immunodeficiency virus type 1 RNA (Ojwang *et al.*, *Proc. Natl. Acad. Sci. USA 89*:10802-10806 (1992)). Other self-cleaving RNA activities are associated with hepatitis delta virus (Kuo *et al.*, *J. Virol. 62*:4429-4444 (1988)).

As discussed above, preferred targets for NTP ribozymes are the nucleotide sequences which are not homologous with PTP sequences. Preferably, the NTP ribozyme molecule of the present invention is designed based upon the chloramphenicol acetyltransferase ribozyme or hairpin ribozymes, described above. Alternatively, NTP ribozyme molecules are designed as described by Eckstein *et al.* (International Publication No. WO 92/07065) who disclose catalytically active ribozyme constructions which have

increased stability against chemical and enzymatic degradation, and thus are useful as therapeutic agents.

In an alternative approach, an external guide sequence (EGS) can be constructed for directing the endogenous ribozyme, RNase P, to intracellular NTP mRNA, which is subsequently cleaved by the cellular ribozyme (Altman *et al.*, U.S. Patent No. 5,168,053). Preferably, the NTP EGS comprises a ten to fifteen nucleotide sequence complementary to an NTP mRNA and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine (*Id.*). After NTP EGS molecules are delivered to cells, as described below, the molecules bind to the targeted NTP mRNA species by forming base pairs between the NTP mRNA and the complementary NTP EGS sequences, thus promoting cleavage of NTP mRNA by RNase P at the nucleotide at the 5'side of the base-paired region (*Id.*).

Included as well in the present invention are pharmaceutical compositions comprising an effective amount of at least one NTP ribozyme or NTP EGS of the invention in combination with a pharmaceutically acceptable carrier. Preferably, the NTP ribozyme or NTP EGS is coadministered with an agent which enhances the uptake of the ribozyme or NTP EGS molecule by the cells. For example, the NTP ribozyme or NTP EGS may be combined with a lipophilic cationic compound which may be in the form of liposomes, as described above. Alternatively, the NTP ribozyme or NTP EGS may be combined with a lipophilic carrier such as any one of a number of sterols including cholesterol, cholate and deoxycholic acid. A preferred sterol is cholesterol.

The NTP ribozyme or NTP EGS, and the pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intra-peritoneal, or transdermal routes. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, as much as 700

milligrams of antisense oligodeoxynucleotide has been administered intravenously to a patient over a course of 10 days (i.e., 0.05 mg/kg/hour) without signs of toxicity (Sterling, "Systemic Antisense Treatment Reported," *Genetic Engineering News 12(12)*:1, 28 (1992)).

Compositions within the scope of this invention include all compositions wherein the NTP ribozyme or NTP EGS is contained in an amount which is effective to achieve inhibition of proliferation and/or stimulate differentiation of the subject cancer cells, or alleviate AD. While individual needs vary, determination of optimal ranges of effective amounts of each component is with the skill of the art.

In addition to administering the NTP antisense oligonucleotides, ribozymes, or NTP EGS as a raw chemical in solution, the therapeutic molecules may be administered as part of a pharmaceutical preparation containing suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the NTP antisense oligonucleotide, ribozyme, or NTP EGS into preparations which can be used pharmaceutically.

Suitable formulations for parenteral administration include aqueous solutions of the NTP antisense oligonucleotides, ribozymes, NTP EGS in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

Alternatively, NTP antisense RNA molecules, NTP ribozymes, and NTP EGS can be coded by DNA constructs which are administered in the form of virions, which are preferably incapable of replicating *in vivo* (*see*, for example, Taylor, WO 92/06693). For example, such DNA constructs may be

administered using herpes-based viruses (Gage *et al.*, U.S. Patent No. 5,082,670). Alternatively, NTP antisense RNA sequences, NTP ribozymes, and NTP EGS can be coded by RNA constructs which are administered in the form of virions, such as retroviruses. The preparation of retroviral vectors is well known in the art (*see*, for example, Brown *et al.*, "Retroviral Vectors," in *DNA Cloning: A Practical Approach*, Volume 3, IRL Press, Washington, D.C. (1987)).

Specificity for gene expression in the central nervous system can be conferred by using appropriate cell-specific regulatory sequences, such as cell-specific enhancers and promoters. For example, such sequences include the sequences that regulate the oligodendroglial-specific expression of JC virus, glial-specific expression of the proteolipid protein, and the glial fibrillary acidic protein genes (Gage *et al., supra*). Since protein phosphorylation is critical for neuronal regulation (Kennedy, "Second Messengers and Neuronal Function," in *An Introduction to Molecular Neurobiology*, Hall, Ed., Sinauer Associates, Inc. (1992)), protein kinase promoter sequences can be used to achieve sufficient levels of NTP gene expression.

Thus, gene therapy can be used to alleviate AD by inhibiting the inappropriate expression of a particular form of NTP. Moreover, gene therapy can be used to alleviate AD by providing the appropriate expression level of a particular form of NTP. In this case, particular NTP nucleic acid sequences may be coded by DNA or RNA constructs which are administered in the form of viruses, as described above. Alternatively, "donor cells" may be modified *in vitro* using viral or retroviral vectors containing NTP sequences, or using other well known techniques of introducing foreign DNA into cells (*see*, for example, *Sambrook et al., supra*). Such donor cells include fibroblast cells, neuronal cells, glial cells, and connective tissue cells (Gage *et al., supra*). Following genetic manipulation, the donor cells are grafted into the central nervous system and thus, the genetically-modified cells provide the therapeutic form of NTP (*Id.*).

Moreover, such virions may be introduced into the blood stream for delivery to the brain. This is accomplished through the osmotic disruption of the blood brain barrier prior to administration of the virions (*see*, for example, Neuwelt, United States Patent No. 4,866,042). The blood brain barrier may be disrupted by administration of a pharmaceutically effective, nontoxic hypertonic solution, such as mannitol, arabinose, or glycerol (*Id.*).

The following clones in *E. coli* were deposited according to the Budapest Treaty with the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, 20852): G2-2 PstI-DH5 (ATCC No. 69257); G5d-PstI-DH5 (ATCC No. 69258); 1-9a-LX-1 blue (ATCC No. 69259); AD3-4-DH1 (ATCC No. 69260); HB4-XL-blue (ATCC No. 69261); AD10-7-DH1 (ATCC No. 69262); AD2-2-DH1- (ATCC No. 69263); G5d-1PstI-EcoRI-DH5 (ATCC No. 69264); and G2-2PstI-EcoRI-DH5 (ATCC No. 69265).

Having now generally described the invention, the same will be more readily understood through reference to the following Examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Example 1

Expression of NTP Immunoreactivity in Cell Lines

Seven cell lines of central nervous system origin were identified that express thread protein immunoreactivity using the Th9 monoclonal antibody which was generated to the pancreatic form of the protein (Gross *et al.*, *J. Clin. Invest. 76*:2115-2126 (1985)), but cross-reacts with thread proteins present in brain tissue and cerebrospinal fluid (Ozturk *et al.*, *Proc. Natl. Acad. Sci. USA 86*:419-423 (1989); de la Monte *et. al.*, *J. Clin. Invest. 86*:1004-1013 (1990); de la Monte *et. al.*, *J. Neurol. Sci. 113*:152-164 (1992); de la Monte *et al.*, *Ann. Neurol. 32*:733-742 (1992)). Among them were the following: two primitive neuroectodermal tumor (PNET) cell lines designated PNET1 and PNET2; three glioblastoma cell lines Hgl 16, Hgl 17, and C6;

the A172 glial cell line; and the SH-Sy5y neuroblastoma cell line. The glioblastoma cell lines and the A172 cells were obtained from the American Type Culture Collection (ATCC). SH-Sy5y cells were obtained from Dr. Biedler at Sloan-Kettering Memorial Hospital. The PNET cell lines have been described previously (The *et al.*, *Nature genetics 3*:62-66 (1993)), and were obtained from Dr. Rene' Bernards at the MGH Cancer Center. All cell lines were maintained in Earl's Modified Eagle Medium supplemented with 10% fetal calf serum, and without antibiotics.

To examine the cells for thread protein and other immunoreactivities, the cultures were harvested in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.4 mM KH_2PO_4, pH 7.3) containing 2 mM EDTA, and cytospin preparations were made using 10^5 cells per slide. The cytospin preparations were fixed immediately in 100% methanol (-20°C), air-dried, and then stored at -80°C until used. Prior to immunostaining, the slides were equilibrated to room temperature and hydrated in PBS. Nonspecific antibody binding was blocked with 3% nonimmune horse serum. Replicate cytospin preparations from the same cultures were incubated overnight at 4°C with 5 or 10 μg/ml of primary antibody. Immunoreactivity was revealed by the avidin-biotin horseradish peroxidase method using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol, and with 3-3′ diaminobenzidine (0.5 mg/ml plus 0.03% hydrogen peroxide) as the chromogen. The cells then were counterstained with hematoxylin, dehydrated in graded alcohol solutions, cleared in xylenes, and preserved under coverglass with Permount (Fisher Scientific).

Cytospin preparations of each cell line were immunostained with the thread protein monoclonal antibodies Th9, Th7, Th10, Th29, Th34, TH46, Th67, and Th90. In addition, replicate slides were immunostained with positive (neurofilament, glial fibrillary acidic protein (GFAP), and vimentin) and negative (desmin, Hepatitis B surface antigen-5C3) control monoclonal antibodies. Except for 5C3 which was generated in the inventor's laboratory

(Fujita *et al.*, *Gastroenterology 91*:1357-1363 (1986)), the control antibodies were purchased (Boehringer-Mannheim). All serological reagents were diluted in PBS containing 1% bovine serum albumin (BSA), and all incubations except the one with primary antibody were carried out at room temperature in humidified chambers. The slides were washed in 3 changes of PBS between each step.

Both PNET1 and PNET2 cells expressed high and middle molecular weight neurofilament proteins and little or no glial fibrillary acidic protein or vimentin. The PNET1, PNET2, and SH-Sy5y cells expressed GAP-43, an abundant calmodulin-binding phosphoprotein that is highly expressed in immature neurons and in neurons undergoing regenerative cell growth (Benowitz *et al.*, *J. Neurosci. 3*:2153-2163 (1983); DeGraan *et al.*, *Neurosci. Lett. 61*:235-241 (1985); Kalil *et al.*, *J. Neurosci. 6*:2563-2570 (1986)). The A172 and C6 cells expressed GFAP and vimentin. However, A172 also exhibited neurofilament immunoreactivity, raising doubt about its purely glial cell nature. None of the cell lines manifested immunoreactivity with monoclonal antibodies to desmin or to Hepatitis B surface antigen. As a negative control cell line, the Huh7 hepatocellular carcinoma cell line was similarly immunostained, and found not to exhibit any immunoreactivity with the above antibodies. However, the Huh cells were immunoreactive with monoclonal antibodies to the insulin receptor substrate protein, IRS-1 (data not shown) which was used as a positive control for this cell line (Sasaki *et al.*, *J. Biol. Chem. 268*:1-4 (1993)).

Using the Th9 monoclonal antibody, thread protein immunoreactivity was detected in primary PNET (A), primary glioblastoma (F), PNET1 (B), and C6 cells (G), but not in hepatocellular carcinoma cell lines (Figure 1). In addition, Th9 immunoreactivity was detected in histological sections from 8 of the 9 primary human CNS PNETs, and from all 5 of the primary human glioblastomas studied (Figure 1). Although all 5 cell lines exhibited intense immunoreactivity with the Th9 monoclonal antibody, they differed with respect to immunoreactivity for other Th monoclonal antibodies. The

immunostaining reaction generated with the Th10 (C,H), Th7 (D,I), or Th46 monoclonal antibodies was either low-level (C,D) or absent (H,I,E,J) in PNET1 (C-E) and C6 (H-J). PNET2 cells exhibited only low levels of immunoreactivity with Th7 and Th29, and they manifested no immunostaining with the other Th monoclonal antibodies. A172, C6, and SH-Sy5y cells displayed little or no immunoreactivity with Th monoclonal antibodies other than Th9. Huh7 cells exhibited no immunoreactivity with any of the thread protein monoclonal antibodies employed, whereas human pancreatic tissue was immunoreactive with all of the Th antibodies, which had been generated against the purified pancreatic form of thread protein (Gross *et al.*, *J. Clin. Invest.* *76*:2115-2126 (1985)).

Example 2

Analysis of Thread Proteins by Monoclonal Antibody-Based Immunoradiometric Assay (M-IRMA)

Cultured cells were washed in PBS and recovered in PBS containing 2 mM EDTA. The cells were pelleted by centrifugation at 1000 x g for 15 min, and then resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin. The supernatant fractions obtained by centrifugation of the lysates at 14,000 x g for 10 min were used for the Western blot analysis, immunoprecipitation studies, and M-IRMA. Protein concentration was determined by the Lowry colorimetric assay. The samples were stored at -40°C.

M-IRMA is a highly sensitive two- or three-site forward sandwich assay which permits quantitation of picomolar NTP in cell lysates, tissue culture medium, tissue homogenates, and body fluids (Ozturk *et al.*, *Proc. Natl. Acad. Sci. USA* *86*:419-423 (1989); de la Monte *et. al.*, *J. Clin. Invest.* *86*:1004-1013 (1990); de la Monte *et. al.*, *J. Neurol. Sci.* *113*:152-164 (1992);

de la Monte *et al., Ann. Neurol. 32*:733-742 (1992); Gross *et al., J. Clin. Invest. 76*:2115-2126 (1985)). In addition, when combined with SDS-PAGE, M-IRMA can be used to determine molecular size of thread proteins and related species (Ozturk *et al., Proc. Natl. Acad. Sci. USA 86*:419-423 (1989); de la Monte *et. al., J. Clin. Invest. 86*:1004-1013 (1990); de la Monte *et. al., J. Neurol. Sci. 113*:152-164 (1992); de la Monte *et al., Ann. Neurol. 32*:733-742 (1992)). M-IRMA involves capturing the immunoreactive thread proteins present in biological samples using monoclonal antibodies Th7 and Th10 affixed to a solid-phase matrix, and then detecting the captured antigen with a third radiolabeled tracer monoclonal antibody (Th9) to the same protein. Briefly, 1/4" polystyrene beads (Precision Ball, Inc) were coated with one or two monoclonal antibodies to thread proteins (usually Th7 + Th10). Cell lysates or supernatant fractions of tissue homogenates (Ozturk *et al., Proc. Natl. Acad. Sci. USA 86*:419-423 (1989); de la Monte *et. al., J. Clin. Invest. 86*:1004-1013 (1990); de la Monte *et. al., J. Neurol. Sci. 113*:152-164 (1992); de la Monte *et al., Ann. Neurol. 32*:733-742 (1992)) were incubated over night with the coated beads to capture thread proteins present in the samples. The beads were washed 5x in PBS, and then incubated with ^{125}-I labeled Th9 as a tracer to detect the captured thread proteins. The concentration of thread protein in the lysate or tissue homogenate was determined from a standard curve generated with known quantities of purified thread protein. This highly sensitive assay can detect as little as 10 pmol of thread protein in solution. To assay for thread proteins fractionated by SDS-PAGE, the wet gels were sliced at 2 mm intervals, and the proteins were eluted from each fraction into 0.5 ml of PBS by shaking for 24 hours at room temperature. The eluates were assayed directly for thread proteins by M-IRMA.

Corresponding with the widespread immunocytochemical staining of PNET1 cells with Th7, Th10, Th34, and Th29, thread protein immunoreactivity was readily measured in these cells by M-IRMA. In other words, with Th7, Th10, Th34, and Th29 monoclonal antibodies (MoAb) used as capture antibodies, either singularly or with two of them together, and ^{125}-I

labeled Th9 was used as the tracer, similarly high levels of thread protein were measured (Figure 2). In contrast, in PNET2, C6, and A172 cells, which exhibited intense immunoreactivity with Th9, but little or no immunocytochemical staining with the Th monoclonal antibodies that were used to capture antigen, the levels of thread protein detected by M-IRMA were much lower than those measured in the PNET1 cells (Figure 2). Similarly, Huh7 cells, which manifested no immunocytochemical staining with any of the thread protein monoclonal antibodies, had virtually nondetectable levels of thread proteins in the cellular lysates by M-IRMA. The concentrations of thread protein in the cell lysates were computed from a standard curve generated with purified PTP using Th7 and Th10 as capture antibodies. The results expressed as mean S.D. pg/mg of total protein were as follows: PNET1-13.1 $\pm$ 0.39; PNET2-2.06 $\pm$ 0.10; A172-3.38 $\pm$ 0.37; C6-2.52 $\pm$ 0.22; and Huh7-0.34 $\pm$ 0.05.

Example 3

Characterization of Neural Thread Proteins in Tumor Cell Lines

In Western Blot anaylsis, samples containing 100 μg of protein were fractionated by SDS-PAGE, along with pre-labeled molecular weight standards. The proteins were blotted onto nylon membranes (Immobilon-P transfer membrane, Millipore) using a semi-dry transfer apparatus (Integrated Systems). The membranes were washed in Tris buffered saline (TBS; 10 mM Tris, 0.85% sodium chloride, pH 7.5), and then blocked with TBS containing 3% BSA. The blots were incubated overnight at 4°C with ^{125}-I labeled Th9 monoclonal antibody. Unspecifically bound probe was removed by washing the membranes at room temperature in TBS-BSA 3 x 15 min. and 1 x 30 min. The results were analyzed by autoradiography using Kodak XAR film.

To prepare samples for immunoprecipitation studies, one milliliter samples of cell lysate containing approximately 1 mg/ml of protein were used for immunoprecipitation studies. The lysates were initially pre-cleared with non-relevant antibody (5C3 or antidesmin), and then with Protein A sepharose. Thread proteins were immunoprecipitated using 5-10 μg of Th9 and Protein A sepharose (Sasaki *et al., J. Biol. Chem. 268*:1-4 (1993)). The immune complexes collected by centrifugation were resuspended in buffer containing 2% SDS and 10 mM β-mercaptoethanol, and then subjected to SDS-PAGE under denaturing and reducing conditions (*Id.*). Crude cellular lysates (100 μg protein) were analyzed simultaneously. The proteins were blotted onto Immobilon-P membranes and probed with ^{125}-I labeled (*Id.*) Th9 to detect thread proteins and related molecules. Negative control experiments were performed simultaneously using either monoclonal antibodies to Hepatitis B surface antigen (5C3) or to desmin.

Metabolic labeling experiments were performed using monolayers of cells cultured in 100 mm^2 petri dishes. Prior to labeling, the cells were exposed to methionine- and cysteine-free medium for 2 h. The medium was then replaced with 3 ml of DMEM containing 300 μCi each of [^{35}S] methionine or [^{35}S] cysteine. After labeling for 3 hours, the cells were incubated for various intervals with complete medium devoid radiolabeled amino acids and supplemented with 10 mM methionine. Cell lysates were prepared as described above. Thread proteins were immunoprecipitated using the Th9 monoclonal antibody and protein A sepharose, and the immunoprecipitation products were analyzed by SDS-PAGE and film autoradiography.

For the *in vivo* phosphorylation studies, cells cultured as described for metabolic labeling studies were washed twice with TBS and incubated for 2 h with phosphate-free Dulbecco's MEM containing 10% dialyzed fetal calf serum. Then the cells were washed with TBS and incubated for 3 h with the same medium containing 400 μCi/ml of [^{32}P] orthophosphoric acid. The cell lysates were analyzed by immunoprecipitation with thread protein, and both

positive (p36) and negative (desmin) control monoclonal antibodies, followed by SDS-PAGE.

In order to study the glycosylation state of neural thread proteins, cell culture lysates containing approximately 100 μg or protein were subjected to SDS-PAGE, and the fractionated proteins were transferred to Immobilon-P membranes (Millipore). O- and N-glycans were detected by periodate oxidation followed by biotinylation, and then Western blot analysis with a Streptavidin-alkaline phosphatase probe and NBT/BCIP as the colorimetric substrate. The assays were performed using the GlycoTrack Kit (Oxford Glycosystems, Rosedale, NY) according to the protocol provided by the manufacturer.

Th9-immunoreactive proteins were detected in lysates of PNET1, PNET2, SH-Sy5y, C6, and A172 cells by four different methods: Western blot analysis, immunoprecipitation followed by Western blot analysis, metabolic labeling followed by immunoprecipitation, and SDS-PAGE combined with M-IRMA. Western blot analysis of crude cellular lysates using ^{125}I-labeled Th9 demonstrated ~21 kDa bands in the above cell lines (as indicated by the arrow in Figure 3), but the signal intensity was low. In contrast, in lysates of human pancreatic tissue, the expected 17 kDa uncleaved and 14 kDa cleaved forms of pancreatic thread protein were readily detected by Western blot analysis (Figure 3). Thread proteins were not detected in lysates of human hepatocellular carcinoma cell lines. The strikingly greater abundance of thread proteins in pancreatic tissue compared with neuronal and glial cell lines is consistent with a previous finding of 10^6-fold higher levels of thread proteins in pancreas and pancreatic juice compared with brain tissue and cerebrospinal fluid (Ozturk *et al.*, *Proc. Natl. Acad. Sci. USA 86*:419-423 (1989); de la Monte *et. al.*, *J. Clin. Invest. 86*:1004-1013 (1990); de la Monte *et. al.*, *J. Neurol. Sci. 113*:152-164 (1992); de la Monte *et al.*, *Ann. Neurol. 32*:733-742 (1992)). Although one would expect that thread proteins synthesized by PNET and glial cells are secreted as is the case for PTP and NTP, thread proteins

were not detected in the tissue culture medium by Western blot analysis, even after concentrating the medium four- or five-fold by lyophilization.

Th9-immunoreactive thread proteins were more readily detected in PNET and glial cell lines by first immunoprecipitating from the lysates with either Th7+Th10 or Th9, and then performing Western blot analysis using ^{125}I-labeled Th9 (direct) (Figure 3), or unlabeled Th9 with ^{125}I-labeled Protein A (indirect). Both methods demonstrated 21 kDa thread protein-related species, similar to those detected by Western blot analysis. In addition, ~17 kDa bands were also observed in both PNET and glial cells, but the signal was inconsistent and low-level, as determined by Western blot analysis. As negative controls, the Huh7, HepG2, and FOCUS (Lun *et al., In Vitro (Rockville) 20*:493-504 (1984)) human hepatocellular carcinoma cell lines were studied simultaneously under identical conditions, and Th9-immunoreactive proteins were not detected in the cellular lysates.

The molecular sizes of thread proteins present in PNET and glial cells were most prominently demonstrated by metabolical labeling with ^{35}S-methionine or ^{35}S-cysteine, followed by immunoprecipitation using Th9 monoclonal antibody. Monoclonal antibodies to desmin or to hepatitis B surface antigen (5C3) were used as negative controls for immunoprecipitation. In both PNET and glial cell lines, ~26 and ~21 kDa Th9-immunoreactive proteins were detected by SDS-PAGE analysis of the immunoprecipitated products (Figure 4, right panel). In PNET1 cells, the 21 kDa band appeared as a doublet (left panel); the accompanying slightly higher molecular weight species appeared to be less abundant than the dominant band at ~21 kDa. In addition, in both PNET and glial cell lines, there were also ~17 kDa Th9-immunoreactive proteins associated with bands of nearly the same intensity as the ~21 kDa bands. In C6 cells, there were also ~26 kDa, ~14-15 kDa and ~8 kDa Th9-immunoreactive proteins which were not detected in PNET cells (Figure 4, arrows).

The 21 kDa and 17 kDa thread proteins in SH-Sy5y, PNET1, A172, and C6 cells, and their absence in hepatocellular carcinoma cells were also

demonstrated by SDS-PAGE/M-IRMA (Figure 5). Cellular proteins fractionated by SDS-PAGE were eluted from the gels sliced at 2 mm intervals, and assayed directly for thread protein immunoreactivity by M-IRMA using Th7+Th10 as capture antibodies, and ^{125}I-labeled Th9 as the tracer. Despite low levels, two distinct peaks were evident in all neuroectodermal cell lines, but not in Huh7 hepatocellular carcinoma cells assayed simultaneously and in the same manner. The resolution of these gels did not permit distinction of ~17 kDa from ~14-15 kDa proteins which might have been present.

PNET1 and C6 cells were metabolically labeled with ^{32}P or ^{35}S-methionine, and thread proteins were immunoprecipitated from the lysates using Th9 monoclonal antibody (Figure 6). As a negative control, immunoprecipitation studies were conducted using an equal portion of the cellular lysate and monoclonal antibodies to desmin protein (Figure 6, right panel). In the cells labeled with ^{35}S methionine, Th9-immunoreactive bands were detected at ~26 kDa and ~21 kDa (upper arrows), ~17 kDa (lower arrows), and also at ~14-15 kDa (Figure 6). After ^{32}P labeling, only the 21 kDa band was observed by immunoprecipitation with Th9 monoclonal antibody; the other molecular weight species did not appear to be phosphorylated (Figure 6). Phosphorylated Th9-immunoreactive proteins were detected in C6 cells, but not in PNET1 cells, but this might be due to less efficient labeling since PNET1 cells grow slower than C6 cells. No bands in the 14 kDa to 26 kDa range were detected using monoclonal antibodies to desmin for immunoprecipitation (Figure 6). Carbohydrate moieties were not detected in Th9 immunoprecipitated proteins (data not shown).

The highest concentrations of thread protein were measured in subconfluent cultures of PNET1 cells, i.e. during the log phase of growth, and the lowest concentrations in overnight serum-starved cultures (growth arrest) (Figure 7). Cultures that were 100% confluent also had lower levels of thread protein expression compared with proliferating cultures. Huh7 hepatocellular carcinoma cells (negative control) were simultaneously studied using identical culture conditions, but the levels of thread protein remained low throughout.

Surprisingly, there was no change in the degree of thread protein immunocytochemical staining of PNET cells cultured under these various conditions. However, the degree to which the levels of thread proteins changed by M-IRMA measurement may not have been detectable by immunocytochemistry. Nevertheless, the reduction in cellular thread protein content induced by serum starvation was associated with a change in the phenotype of the cells. When the cells achieved 100% confluence or after they had been subjected to overnight serum starvation, the cell bodies reduced in size, and they exhibited striking changes in the degree and distribution of immunoreactivity for neurofilament protein, GAP-43, and GFAP (Figure 8). In PNET cultures that were 50% confluent, the cells exhibited punctate and often a polar distribution of neurofilament and GAP-43 immunoreactivity, whereas 100% confluent and serum-starved PNET cultures exhibited diffuse perikaryal immunoreactivity for both neurofilament and GAP-43. The punctate immunoreactivity may have corresponded with distribution of neurofilament and GAP-43 in neurites. In contrast, 50% confluent PNET cultures were devoid of GFAP immunoreactivity, while 100% confluent and serum-starved cultures contained conspicuous proportions of GFAP-positive cells. Moreover, the proportion of GFAP-immunoreactive cells was greatest in 100% confluent serum-starved cultures, followed by 50% confluent serum-starved cultures, and then 100% confluent cultures with medium containing 10% fetal calf serum. Therefore, the reduction in thread protein levels measured in PNET cells subjected to overnight serum starvation may have been due to differentiation of the cells toward an astrocytic phenotype. C6 cells and other glioblastoma cell lines exhibited intense immunoreactivity with the Th9 monoclonal antibody, but the levels of thread protein measured by M-IRMA were often low, possibly due to low-level immunoreactivity with other thread protein antibodies, including Th7 and TH10 (*see* Figure 1).

Example 4
Cloning of Thread Proteins from Human cDNA Libraries

Human brain cDNA libraries made from 17-18 week old fetal brain (Stratagene, Inc., La Jolla, CA), 2 year-old temporal lobe neocortex (Stratagene), and end-stage Alzheimer's disease cerebral cortex (In Vitrogen; San Diego, CA) were screened using probes generated from a 416 bp DNA fragment corresponding to nucleotides 235-650 of the rat PTP cDNA. The rat PTP cDNA, designated O18, was isolated from a rat pancreatic cDNA library using synthetic 60mer DNA probes corresponding to nucleotides 45-104 and 345-404 of the published sequence (Terazono *et al.*, *J. Biol. Chem. 263*:2111-2114 (1988); Watanabe *et al.*, *J. Biol. Chem. 265*:7432-7439 (1990)). Approximately 2 x 10^6 plaques or colonies from each library were screened with low-stringency hybridization using standard techniques (*see* Sambrook *et al.*, *supra*). Putative clones were plaque/colony purified, and the DNA inserts were sequenced by the dideoxynucleotide chain termination method using T7 polymerase (USB Sequenase; United States Biochemical Corp., Cleveland, OH). The sequences were compared with the Genebank database, and aligned with the nucleic acid sequences of other thread protein cDNAs.

1. CNS Neural Thread Protein cDNA Isolated from Human Fetal Brain Library

A 1.35 kilobase (kb) 1-9a CNS thread protein partial cDNA was isolated in which only a small segment corresponds to an open reading frame, and the remainder, to a 3′ untranslated region (Figure 9a). The sequence of an additional 150 nucleotides was obtained from 5′ anchor PCR amplification products. A second round of 5′ anchor PCR amplification yielded a further upstream 600 bp product (Figure 9b). A portion of the 1-9a cDNA sequence shares significant homology with the 5′ end of the human PTP cDNA and the Reg gene (Figure 10a). In addition, the initial 5′ anchor PCR product has

60% homology with the 5' end of the Reg gene, and 63% homology with Exon 2 of the human Reg gene (Figure 10b). Moreover, probes generated from the 590 bp 5'-end fragment of 1-9a cDNA hybridized with human brain and pancreas mRNA (Figure 12). The 1-9a sequence is also homologous with the AD2-2 and AD3-4 cDNAs in that at one end of their completed sequences, the overlaps are substantial (Figure 10c).

2. *CNS Neural Thread Protein cDNA Isolated from a Two-Year Old Temporal Cortex Library*

The HB4 clone is a 593 base pair partial cDNA that was isolated from a 2-year old temporal cortex library. This cDNA contains an open reading frame at its 5' end and terminates at nucleotide 275. There is a polyadenylation signal beginning at nucleotide 475, and the sequence ends with a poly-A tail (Figure 11a). The deduced amino acid sequence of the partial HB4 clone predicts a protein with a molecular weight of 10.4 kDa, and a pI of 12.1. The HB4 cDNA exhibits 50% overall nucleic acid homology with the human PTP cDNA (Figure 11b), a segment of the human Reg gene (Figure 11c).

3. *Isolation of Neural Thread Protein cDNAs from an Alzheimer's Disease Library*

Using the O18 rat PTP cDNA probe, four related cDNAs were isolated from an AD brain library. These clones were designated: AD 2-2, AD 3-4, AD 4-4 and AD 16c (also called AD 10-7) (Figures 16a-16l).

The AD 2-2 cDNA is approximately 1.2 kb and it shares significant homology with the 1-9a cDNA, AD 16c, rat PTP cDNA, and Exon 1 of the human Reg gene (Figure 17). The AD 2-2 probe generates a genomic Southern blot pattern similar to that obtained with the AD 3-4 probe. Figure 16b depicts the complete nucleotide sequence of the AD2-2 cDNA clone that

was isolated from an AD brain library. Random primer generated probes based on this sequence hybridized with human brain and neuronal samples but not with glial cell lines of with pancreatic RNA.

Figures 16c, 16d, 16e and 16f depict partial nucleotide sequences of the AD3-4 cDNA clones that were isolated from an AD brain library. Rnadom primer generated AD3-4 probes yielded two mRNA transcripts, 1.6 kB and 3.4 kB. These mRNA species are over-expressed in AD brains, with an average of two-fold elevation compared with aged matched controls (N=8).

The AD 3-4 cDNA 1.6 kb clone is identical to another clone isolated at the same time (AD 5-3) (Figure 18a). The AD 3-4/AD 5-3 cDNA exhibits substantial homology with the 1-9a 5' anchor PCR products (Figure 18b), as well as with the human Reg gene and the Gen2a-EP genomic clone (Figure 18c). Southern blot analysis of human genomic DNA with the AD 3-4 probe revealed a pattern similar to that obtained with the AD 2-2 probe.

Figures 16g and 16h depict the partial nucleotide sequence of AD 4-4 which is a 0.8 kb partial cDNA clone which is identical to another cDNA isolated at the same time (AD 3-5). This AD 4-4 clone shares substantial sequence homology with AD 2-2 and 1-9a cDNAs (Figure 19). Figure 16i depicts the complete nucleotide sequence of a partial cDNA clone isolated from an AD brain library. This cDNA hybridized with brain and neuronal cell line mRNA, yielding a single 1.4 kB transcript.

Figure 16j depicts the nucleotide sequence of the 0.5 kb partial cDNA clone AD 16c (also called AD 10-7) that is 72% homologous with AD 2-2, and also aligns with human PTP and the human Reg gene sequences (Figures 20a and b).

Figure 16k depicts the complete nucleotide sequence of the AD10-7 clone that was isolated from an AD brain library. Hybridization of Northern blots using either antisense cRNA probes or random primer generated DNA probes detected 2.6, 1.9. 1.4 and 0.9 kB mRNA transcripts in neuronal cells. Neuronal cell lines expressed only the two largest transcripts, while mature adult human brains expressed predominantly the two smallest transcripts, and

either very low or nondetectable levels of the 2.6 kB and 1.9 kB transcripts. Using an AD10-7 probe, Northern blot analysis of RNA obtained from human liver, ovary, fallopian tube, colon, stomach, spleen, rectum, thyroid, 12 week placenta and kidney was negative.

Figure 16l depicts the complete nucleotide sequence of the AD16c cDNA clone that was isolated from an AD brain library. Hybridization of Northern blots using random primer generated DNA probes yielded the same results as obtained with the AD10-7 cDNA clone. The AD16c clone shares a 650 bp segment of near identity with AD10-7. In addition, elevated levels of AD16c mRNA were detected in AD brains compared with aged control brains by Northern blot analysis.

Example 5

Analysis of Brain Thread Protein Gene Expression

Thread protein mRNA expression was examined in the following neuroectodermal tumor derived cell lines: central nervous system primitive neuroectodermal tumor cells designated PNET1 and PNET2; HGL-16 and HGL-17 human glioblastoma cells; A172 human glioma cells; C6 rat glioma cells; and SH-Sy5y neuroblastoma cells. In addition, human brain tissue from patients with Alzheimer's disease or no neurological disease (aged controls), and embryonic and postnatally developing rat brain were assayed for thread protein mRNA expression. RNA extracted from human and rat pancreas served as positive controls.

RNA was extracted in 5 M guanidinium isothiocyanate, and then isolated by centrifugation through a cesium chloride step gradient (*see* Sambrook *et al., supra*). RNA was quantified by measuring the absorbance at 260 nm and 280 nm. The thread protein mRNA transcript sizes were assessed by northern blot analysis, and the levels of expression were evaluated by RNA dot blot hybridization. Northern blot analysis was performed by electrophoresing samples containing 15 μg of total cellular RNA through 1% agarose-formaldehyde gels. The RNA was transferred to nylon membrane, cross-linked with ultraviolet light, and hybridized with probes generated from a 600 bp fragment of the 1-9A cDNA clone. The fragment used for hybridization studies contained the regions most homologous with the human PTP cDNA. The probes were labeled with [^{32}P] α-dCTP by the random primer method (Amersham Corporation; Arlington Heights, IL). The blots were hybridized overnight at 42°C with 2 x 10^6 dpm /ml of probe in buffer containing 50% formamide, 5x SSPE, 10x Denhardt's (100x Denhardt's is 2% Ficoll, 2% bovine serum albumin, 2% polyvinylpyrollidine), 0.5% SDS (sodium dodecyl sulfate), and 100 μg/ml of sheared denatured salmon sperm DNA. The membranes were washed in SSPE containing 0.25% SDS using

standard methods. Autoradiograms were generated by exposing the membranes to Kodak XAR film at -80°C. The membranes were subsequently stripped of probe and then rehybridized with a synthetic 30mer corresponding to 18s RNA to evaluate sample loading.

Northern analysis of total cellular RNA using probes made from the 1-9a cDNA disclosed two dominant transcripts in central nervous system (CNS) tumor cell lines: one transcript was 1.6 kb, and the other was 0.9 kb (Figure 12A). In addition, in the SH-Sy5y neuroblastoma and PNETI cell lines, a larger 4.2 kb mRNA transcript was also detected. The 4.2 kb transcript may represent preprocessed mRNA. The same size transcripts were detected in adult (R. Brain) and newborn (NB) rat, but the 0.9 kb transcript was more abundant in the adult brain whereas the 1.6 kb transcript was more abundant in the newborn rat brain. In rat pancreas (R. Panc.), only a 0.9 kB transcript was detected, corresponding to the size of rat PTP mRNA (Terazono *et al.*, *J. Biol. Chem. 263*:2111-2114 (1988); Watanabe *et al.*, *J. Biol. Chem. 265*:7432-7439 (1990)). mRNA transcripts were not detected in normal liver (Nl Liver). Using a probe generated from the 3' region of the 1-9a cDNA, the 1.6 kb, but not the 0.9 kb transcript was revealed (Figure 12b). Using a 30-mer probe corresponding to the most 5'-end of the 1-9a cDNA, the higher molecular weight mRNA transcripts were detected (Figure 12c). The 0.9 kb transcript was also evident with longer exposure of the blot.

Northern analysis of human brain RNA disclosed a dominant 1.6 kb transcript, but also two and sometimes three smaller transcripts of 1.2 kb, 0.9 kb, and 0.8 kb (Figure 13, bottom). In contrast to the findings in cell lines, the 4.2 kb mRNA transcript was seldom observed in adult human brain. Hybridization with human pancreas disclosed a 0.8 kb transcript, corresponding with the size of PTP mRNA. The transcripts detected in human brain and pancreas using 1-9a probes were identical in size to the transcripts observed using PTP cDNA probes.

Dot blot RNA hybridization to 5 μg of total RNA using the 600 bp fragment of the 1-9a cDNA (NTP) demonstrated higher levels of expression

in AD, compared with aged control brains (Figure 13, top). Rehybridization of the same membrane with a cDNA corresponding to β-actin demonstrated similar loading of RNA in each dot. The observation of elevated levels of 1-9a-related mRNA in AD brain tissue is similar to that reported previously using 60mer probes corresponding to human PTP cDNA (de la Monte *et. al.*, *J. Clin. Invest. 86*:1004-1013 (1990)). The differences between AD and control brains appeared to be due to differences in the levels of the 1.6 kb, 0.9 kb and 0.8 kb transcripts, as shown in Figure 13.

The AD-NTP 3-4 cDNA, isolated from the AD library, hybridizes with RNA from neuronal-derived neuroectodermal tumor cell lines, and human brain tissue. In the cell lines, 1.6 kb and 0.9 kb transcripts as observed with the 1-9a probe were detected (Figure 21c). However, in human brain, ~4 kb, 1.6 kb, and 0.9 kb transcripts were detected, and the levels of expression for all three transcripts were higher in AD compared with aged control brains (Figure 21d).

AD 4-4 cDNA probe hybridized only with a 0.9 kb transcript, and only in neuronal cell lines.

Example 6

Direct Cloning and Sequencing of Thread Protein cDNAs from Neuroectodermal Tumor Cell Lines and Alzheimer's Disease Brain

Thread protein cDNAs were cloned directly from PNET1, PNET2, SH-Sy5y, and A172 cells, and from Alzheimer's disease and aged control brain RNA using the 3'- and 5'-RACE methods (Frohman *et al., Proc. Natl. Acad. Sci. USA 85*:8998 (1988); Ohara *et al., Proc. Natl. Acad. Sci. USA 86*:5673 (1989); Loh *et al., Science 243*:217 (1989)). Briefly, RNA was reverse transcribed using oligo-dT primers. For the 5'-RACE reaction, the cDNAs were amplified by polymerase chain reaction (PCR) using a specific 17-mer corresponding to a 5'-region of the 1-9a sequence, and a 17 dT

primer. The resulting PCR products were subjected to another round of amplification using another internal but overlapping 5'-end primer, and a specific 3'-17-mer corresponding to a 3' region of the 1-9a sequence. For the 3'-RACE reactions, the cDNAs were first tailed with dCTP using terminal deoxynucleotide transferase, and then they were amplified using a specific 17-mer corresponding to nucleotides 781-797 of the 1-9a clone and dG (17mer). A second nested PCR amplification was performed using a specific 17mer corresponding to nucleotides 766-792 at the 3' end, and dGTP (17mer) for the 5' end. The PCR products were subjected to Southern blot analysis using probes generated from an internal DNA fragment of the 1-9a cDNA clone, and from the O18 rat PTP cDNA clone. The PCR products were gel purified and ligated into pAmpl vectors using uracil deoxytransferase. The subcloned DNA inserts were sequenced by the dideoxynucleotide chain termination method using T7 DNA polymerase.

CNS thread protein transcripts were detected in neuroectodermal tumor cell lines and in AD human brain tissue by reverse transcription followed by PCR using specific primers corresponding to the 5' and 3' regions of the 1-9a cDNA sequence. Southern blot analysis of the PCR products demonstrated two dominant cross-hybridizing species, 0.8 kb and 1.0 kb (Figures 14a and 14b). In addition, in the SH-Sy5y cells, a larger 1.8 kb PCR product was also detected. In the PNET1, PNET2, SH-Sy5y, and A172 cells, a 0.4 kb PCR product that hybridized with the 1-9a probe was observed. Corresponding with the higher levels of thread protein mRNAs in Alzheimer's disease brains, the hybridization signal was more intense in AD samples compared with aged control samples.

The PCR products generated from the SH-Sy5y cells were subcloned and sequenced. Southern analysis of the cloned fragments exhibited intense hybridization with the 1-9a cDNA, and less intense but definite hybridization with the O18 cDNA (rat PTP) (Figure 14c). The nucleic acid sequence of the SH-Sy5y PCR clone (Sy-NTP) was identical to the 1-9a cDNA sequence.

Example 7

Isolation of Genomic Clones Coding for Human Brain Thread Proteins

A human genomic DNA library was screened using probes made with a 600 bp fragment of the 1-9a human brain thread protein cDNA that was isolated from the two year-old temporal cortex library. The 1-9a cDNA fragment contained a region with 60% nucleic acid sequence homology with human PTP. After colony purification, the putative genomic clones were checked for cross-hybridization with the O18 rat PTP cDNA fragment. *Eco*RI, *Pst*I, and *Eco*RI/*Pst*I restriction fragments that hybridized with both the 1-9a and O18 probes were subcloned into pBluescript II vectors (Promega, Inc., Madison, WI) and then sequenced by the dideoxynucleotide chain termination method using either T7 polymerase (USB Sequenase) or polymerase chain reaction amplification and Vent polymerase.

Four genomic fragments designated G2-2 *Pst*I, G2-2 *Pst*I-*Eco*RI, G5d-1 *Pst*I, and G5d-1 *Pst*I-*Eco*RI were isolated from a human genomic DNA library (Figures 22a-22d). These genomic fragments all hybridized with both the 1-9a and O18 cDNA probes, and they ranged in size between 1.5 kb and 3 kb. Partial nucleic acid sequence information demonstrated homology between G2-2*Pst*I and the human Reg gene and human and rat PTP cDNAs (Figure 23a); between G2-2 *Pst*I-*Eco*RI and both the Reg gene and rat PTP cDNA (Figure 23b), and also with AD 2-2, AD 3-4, and the 1-9a cDNAs (data not shown); between G5d-1 *Pst*I and the Reg gene and human PTP (Figure 23c); and between G5d-1 *Pst*I-*Eco*RI and Reg gene, human PTP, 1-9a, and AD 4-4.

Example 8

In vitro Expression of the LacZ Fusion Protein and Demonstration of its Relatedness to Thread Proteins

Fusion protein expression in bacteria containing the 1-9a cDNA clone, or one of the four genomic clones was induced with isopropylthio-ß-D-galactoside (IPTG) using standard techniques (Sambrook *et al.*, *supra*). Crude bacterial lysates from induced and uninduced cultures were subjected to SDS-PAGE and Western blot analysis using the Th9 monoclonal antibody to thread protein (Sasaki *et al.*, *J. Biol. Chem. 268*:1-4 (1993)), and ^{125}-I labeled protein A to detect the bound antibody. In addition, bacterial lawns containing cloned DNA were induced to express the fusion protein with IPTG, and replica filters were probed directly with Th9 monoclonal antibody followed by ^{125}-I labeled protein A.

Thread protein immunoreactivity was demonstrated in the bacterial fusion proteins by direct antibody binding to the IPTG-induced colonies (Figure 24). Thread protein immunoreactivity was detected using a cocktail of Th9, Th7, and Th10 monoclonal antibodies to PTP (Sasaki *et al.*, *J. Biol. Chem. 268*:1-4 (1993), and ^{125}-I labeled Protein A.

Example 9

Relative Levels of AD16c mRNA in AD and Aged Control Brains

Northern blot analysis was performed using an AD16 cDNA probe. The blots were re-probed to detect 18s ribosomal RNA to evaluate loading of RNA in each lane. The unsaturated autoradiograms were subjected to densitometric analysis using a Molecular Dynamics Image Analyzer. The ratios of the AD16c and 18s RNA hybridization signals were plotted for each case, and the results are depicted graphically in Figure 25. The mean ratios (relative levels of AD16c) with standard errors are depicted in the smaller

right hand graph. The findings confirm that there are elevated levels of AD16c mRNA expression in 6 of 9 AD brains compared to 1 of 6 age-matched controls. The difference between the mean levels is highly statistically significant ($P < 0.005$). Similar results were obtained using AD10-7 probes. Theses results demonstrate that there is a statistically significant increase in levels of expression in AD brains compared to control brains.

Example 10

Preparation of Recombinant AD10-7 Fusion Protein and Detection Thereof With Monoclonal Antibodies

AD10-7 cDNA was ligated into pTrcHIS vectors (In Vitrogen, San Diego) in three different reading frames (two incorrect-A and B, and one correct-C). Bacteria transformed with one of the three plasmids were induced with IPTG and bacterial lysates were examined for protein expression 0, 1 and 5 hours later. The proteins were fractionated by SDS-PAGE, and Western blot analysis was performed using monoclonal antibodies against the expressed tag protein (T7-tag mouse monoclonal antibodies; Novogen). The blots were developed using the avidin-biotin, horseradish peroxidase method, with diaminobenzidine as the chromogen (Figure 26). A band corresponding to ~45 kDA was detected in bacteria that had been transformed with plasmid DNA which contained AD10-7 ligated only in the correct reading frame (C) (arrow). The same size protein was observed by *in vitro* translation of the AD10-7 cDNA in a rabbit reticulocyte lysate assay system. In both systems, the fusion partner peptide was ~3 kDA, indicating that the cDNA encodes a protein of about ~42 kDA. A ~42 kDA NPT species is routinely detected by Western Blot analysis of neuronal cell lines and of human brain tissue.

Example 11

Demonstration of Neuronal Localization of AD10-7 mRNA Expression by In Situ Hybridization

Sense and antisense cRNA probes were generated from linearized AD10-7 plasmid DNA using SP6 or T7 DNA-dependent RNA polymerase, respectively. The antisense probes hybridized with neuronal cell line mRNA as described above for this clone. The cRNA sense probes, on the other hand, failed to hybridize with RNA by Northern blot analysis. cRNA probes labeled with digoxigenin-UTP were hybridized with human brain tissue sections from early AD. After washing the sections extensively (de la Monte *et al.*, *J. Clin. Invest.* *86*:1004-1013 (1990)), the hybridized probes were detected using peroxidase or alkaline phosphatase conjugated monoclonal antibodies to digoxigenin, and the colorimetric reactions were revealed using standard methods. Examination of the sections by brightfield and darkfield microscopy demonstrated hybridization of AD10-7 only in neurons (Fig. 27; dense aggregates of white grains over cell bodies in (A)). In contrast, and similar to the findings by Northern blot analysis, the *sense* AD10-7 cRNA probes failed to hybridize with brain tissue (B).

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following Claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent

application was specifically and individually indicated to be incorporated by reference in their entirety.

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Escherichia coli: G2-2PstI-DH5

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Escherichia coli: G5d-PstI-DH5

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Escherichia coli: 1-9a-LX-1 blue

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Escherichia coli: AD3-4-DH1

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Escherichia coli: HB4-XL-blue

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Escherichia coli: AD10-7-DH1

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Escherichia coli: AD2-2-DH1

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Escherichia coli: G5d-1PstI-EcoRI-DH5

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What Is Claimed Is:

1. A method for detecting the presence of Neural Thread Protein (NTP) having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, in a human subject, said method comprising:

(a) contacting a biological sample from said human subject that is suspected of containing said NTP with at least one molecule capable of binding to said protein; and

(b) detecting any of said molecule bound to said protein.

2. The method of claim 1, wherein said molecule is selected from the group consisting of:

(a) an antibody substantially free of natural impurities;

(b) a monoclonal antibody; and

(c) a fragment of (a) or (b).

3. The method of claim 1, wherein said biological sample is removed from said human subject prior to contacting said sample with said molecule.

4. The method of claim 1, wherein the detecting of any of said molecule bound to said protein is performed by *in situ* imaging.

5. The method of claim 1, wherein the detecting of any of said molecule bound to said protein is performed by *in vitro* imaging.

6. The method of claim 1, wherein said molecule is administered to said human subject.

7. The method of claim 1, wherein said molecule is bound to said protein *in vivo*.

8. A method of diagnosing the presence of Alzheimer's Disease in a human subject suspected of having Alzheimer's Disease which comprises:

(a) incubating a biological sample from said subject which is suspected of containing NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, in the presence of at least one binding molecule capable of identifying said NTP; and

(b) detecting said binding molecule which is bound in said sample, wherein said detection indicates that said subject has Alzheimer's Disease.

9. The diagnostic method of claim 8, wherein said detection is by immunometric assay.

10. The diagnostic method of claim 9, wherein said immunometric assay is a monoclonal antibody-based immunometric assay.

11. The diagnostic method of claim 8, wherein said method comprises:

(a) incubating said biological sample with two different NTP monoclonal antibodies bound to a solid phase support; and

(b) detecting NTP bound to said monoclonal antibodies with a third different detectably labeled NTP monoclonal antibody in solution.

12. The diagnostic method of claim 8, wherein said incubating step further includes adding a known quantity of labeled Neural Thread Protein whereby a competitive immunoassay is established.

13. The diagnostic method of claim 12, wherein said label is capable of emitting radiation.

14. The diagnostic method of claim 13, wherein said label is ^{125}I.

15. The diagnostic method of claim 8, wherein said detection is by immuno-polymerase chain reaction.

16. A method of diagnosing the presence of neuroectodermal tumors in a human subject suspected of having a neuroectodermal tumor which comprises:

(a) incubating a biological sample from said subject which is suspected of containing NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, in the presence of at least one binding molecule capable of identifying said NTP; and

(b) detecting said binding molecule which is bound in said sample, wherein said detection indicates that said subject has a neuroectodermal tumor.

17. The diagnostic method of claim 16, wherein said detection is by an immunometric assay.

18. The diagnostic method of claim 17, wherein said immunometric assay is a monoclonal antibody-based immunometric assay.

19. The diagnostic method of claim 16, wherein said method comprises:

(a) incubating said biological sample with two different NTP monoclonal antibodies bound to a solid phase support; and

(b) detecting NTP bound to said monoclonal antibodies with a third different detectably labeled NTP monoclonal antibody in solution.

20. The diagnostic method of claim 16, wherein said incubating step further includes adding a known quantity of the corresponding labeled NTP whereby a competitive immunoassay is established.

21. The diagnostic method of claim 20, wherein said label is capable of emitting radiation.

22. The diagnostic method of claim 21, wherein said label is ^{125}I.

23. The diagnostic method of claim 16, wherein said detection is by immuno-polymerase chain reaction.

24. A method of diagnosing the presence of a malignant astrocytoma in a human subject suspected of having a malignant astrocytoma which comprises:

(a) incubating a biological sample from said subject which is suspected of containing NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, in the presence of at least one binding molecule capable of identifying said NTP; and

(b) detecting said binding molecule which is bound in said sample, wherein said detection indicates that said subject has a malignant astrocytoma.

25. The diagnostic method of claim 24, wherein said detection is by an immunometric assay.

26. The diagnostic method of claim 25, wherein said immunometric assay is a monoclonal antibody-based immunometric assay.

27. The diagnostic method of claim 24, wherein said method comprises:

(a) incubating said biological sample with two different NTP monoclonal antibodies bound to a solid phase support; and

(b) detecting NTP bound to said monoclonal antibodies with a third different detectably labeled NTP monoclonal antibody in solution.

28. The diagnostic method of claim 24, wherein said incubating step further includes adding a known quantity of the corresponding labeled NTP whereby a competitive immunoassay is established.

29. The diagnostic method of claim 28, wherein said label is capable of emitting radiation.

30. The diagnostic method of claim 29, wherein said label is ^{125}I.

31. The diagnostic method of claim 24, wherein said detection is by immuno-polymerase chain reaction.

32. A method of diagnosing the presence of a glioblastoma in a human subject suspected of having glioblastomas which comprises:

(a) incubating a biological sample from said subject suspected of containing NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, in the presence of at least one binding molecule capable of identifying said NTP; and

(b) detecting said binding molecule which is bound in said sample, wherein said detection indicates that said subject suffers from a glioblastoma.

33. The diagnostic method of claim 32, wherein said detection is by an immunometric assay.

34. The diagnostic method of claim 33, wherein said immunometric assay is a monoclonal antibody-based immunometric assay.

35. The diagnostic method of claim 32, wherein said method comprises:

(a) incubating said biological sample with two different NTP monoclonal antibodies bound to a solid phase support; and

(b) detecting NTP bound to said monoclonal antibodies with a third different detectably labeled NTP monoclonal antibody in solution.

36. The diagnostic method of claim 32, wherein said incubating step further includes adding a known quantity of labeled NTP whereby a competitive immunoassay is established.

37. The diagnostic method of claim 36, wherein said label is capable of emitting radiation.

38. The diagnostic method of claim 37, wherein said label is ^{125}I.

39. The diagnostic method of claim 32, wherein said detection is by immuno-polymerase chain reaction.

40. NTP substantially free of any natural impurities and having a molecular weight of about 42 kDa.

41. NTP substantially free of any natural impurities and having a molecular weight of about 26 kDa.

42. NTP substantially free of any natural impurities and having a molecular weight of about 21 kDa.

43. NTP substantially free of any natural impurities and having a molecular weight of about 17 kDa.

44. NTP substantially free of any natural impurities and having a molecular weight of about 14 kDa.

45. NTP substantially free of any natural impurities and having a molecular weight of about 8 kDa.

46. A recombinant DNA molecule comprising a genetic sequence coding for an NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa.

47. The DNA molecule of claim 46 wherein said NTP is human NTP.

48. The DNA molecule of claim 46 which is a plasmid.

49. A host transformed with the plasmid of claim 48.

50. A method of using the plasmid of claim 48 to prepare an NTP, said method comprising:

(a) introducing said plasmid into a host cell to produce a recombinant host cell;
(b) culturing said recombinant host cell; and
(c) isolating said NTP from said recombinant host cell.

51. A DNA probe comprising a detectably labeled genetic sequence coding for an NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, or a fragment thereof.

52. A method of detecting the presence of a genetic sequence coding for NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, in a clinical sample, which comprises:

(a) contacting said sample with the probe of claim 51 under conditions of hybridization; and

(b) detecting the formation of a hybrid of said probe and said sequence.

53. A method of producing an NTP having a molecular weight of about 8 kDa, about 14 kDa, about 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, said method comprising:

(a) culturing a recombinant host comprising a human gene coding for said NTP; and

(b) isolating said NTP from said host.

54. The method of claim 53, wherein said gene is obtained from brain tissue of a patient with Alzheimer's Disease.

55. The method of claim 53, wherein said gene is obtained from a human neural tumor cell line.

56. The method of claim 53, wherein said gene codes for an NTP having a molecular weight of about 42 kDa.

57. The method of claim 53, wherein said gene codes for an NTP having a molecular weight of about 26 kDa.

58. The method of claim 53, wherein said gene codes for an NTP having a molecular weight of about 21 kDa.

59. The method of claim 53, wherein said gene codes for an NTP having a molecular weight of about 17 kDa.

60. The method of claim 53, wherein said gene codes for an NTP having a molecular weight of about 14 kDa.

61. The method of claim 53, wherein said gene codes for an NTP having a molecular weight of about 8 kDa.

62. The method of claim 53, wherein said host is *E. coli*.

63. The method of claim 53, wherein said gene is contained by a vector.

64. The method of claim 53, wherein said gene is under control of an inducible promoter.

65. The method of claim 64, wherein said promoter is a lambda P_L promoter.

66. The method of claim 64, wherein said promoter is a *tac* promoter.

67. A substantially pure NTP having a molecular weight of about 42 kDa obtained by the process of claim 53.

68. A substantially pure NTP having a molecular weight of about 26 kDa obtained by the process of claim 53.

69. A substantially pure NTP having a molecular weight of about 21 kDa obtained by the process of claim 53.

70. A substantially pure NTP having a molecular weight of about 17 kDa obtained by the process of claim 53.

71. A substantially pure NTP having a molecular weight of about 14 kDa obtained by the process of claim 53.

72. A substantially pure NTP having a molecular weight of about 8 kDa obtained by the process of claim 53.

73. A 15- to 30-mer antisense oligonucleotide which is complementary to an NTP nucleic acid sequence and which is nonhomologous to the PTP nucleic acid sequence.

74. The antisense oligonucleotide of claim 73 which is DNA.

75. The antisense oligonucleotide of claim 73 which is an o-oligonucleotide.

76. The antisense oligonucleotide of claim 73 which is an S-oligonucleotide.

77. A pharmaceutical composition comprising at least one 15- to 30-mer antisense oligonucleotide which is complementary to an NTP sequence and which is nonhomologous to the PTP sequence; and a pharmaceutically acceptable carrier.

78. A ribozyme comprising a target sequence which is complementary to an NTP sequence and nonhomologous to the PTP nucleic acid sequence.

79. A DNA molecule which codes for the ribozyme of claim 78.

80. A pharmaceutical composition comprising the NTP ribozyme of claim 78 and a pharmaceutically acceptable carrier.

81. A method for inhibiting the expression of an NTP in a patient, said method comprising administering to said patient an effective amount of the antisense oligonucleotide of claim 73.

82. A method for inhibiting the expression of an NTP in a patient, said method comprising administering to said patient an effective amount of the ribozyme of claim 78.

83. A method for inhibiting the expression of an NTP in a patient, said method comprising administering to said patient an effective amount of the DNA molecule of claim 79.

84. An oligonucleotide comprising the sequence 3'X5'-L-5'X3', wherein X comprises an NTP nucleic acid sequence which is nonhomologous to the PTP nucleic acid sequence, and wherein L represents an oligonucleotide linkage.

85. An oligonucleotide comprising the sequence 5'X3'-L-3'X5', wherein X comprises an NTP nucleic acid sequence which is nonhomologous to the PTP nucleic acid sequence, and wherein L represents an oligonucleotide linkage.

86. A method to treat diseases or conditions mediated by the presence of an NTP having a molecular weight of about 8 kDa, 14 kDa, 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, which method comprises administering to a patient in need of such treatment an effective amount of the oligonucleotide of claims 84 or 85, or a pharmaceutical composition thereof.

87. A ribonucleotide NTP External Guide Sequence comprising:

(a) a 10-15 nucleotide sequence which is complementary to an NTP nucleic acid sequence and which is nonhomologous to the PTP nucleic acid sequence; and

(b) a 3'-NCCA nucleotide sequence, wherein N is a purine.

88. The NTP External Guide Sequence of claim 87 which is DNA.

89. A method to treat diseases or conditions mediated by the presence of an NTP having a molecular weight of about 8 kDa, 14 kDa, 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, which method comprises administering to a patient in need of such treatment an effective amount of the ribonucleotide NTP External Guide Sequence of claim 87, or a pharmaceutical composition thereof.

90. An expression vector comprising the DNA molecule of claim 46.

91. A virion comprising the expression vector of claim 90.

92. A method to treat diseases or conditions mediated by the abnormally low level of expression of an NTP having a molecular weight of about 8 kDa, 14 kDa, 17 kDa, about 21 kDa, about 26 kDa or about 42 kDa, which method comprises administering to a patient in need of such treatment an effective amount of the virion of claim 91.

93. A method of differentiating between sporadic and familial Alzheimer's Disease in a human subject, said method comprising:

(a) obtaining a biological sample from said human subject who is suspected of having Alzheimer's Disease;

(b) purifying DNA from said biological sample; and

(c) contacting said DNA with the probe of claim 51 under conditions of hybridization;

wherein familial Alzheimer's Disease is indicated by the detection of a hybrid of said probe and said DNA, and

wherein sporadic Alzheimer's Disease is indicated by the absence of detection of hybridization.

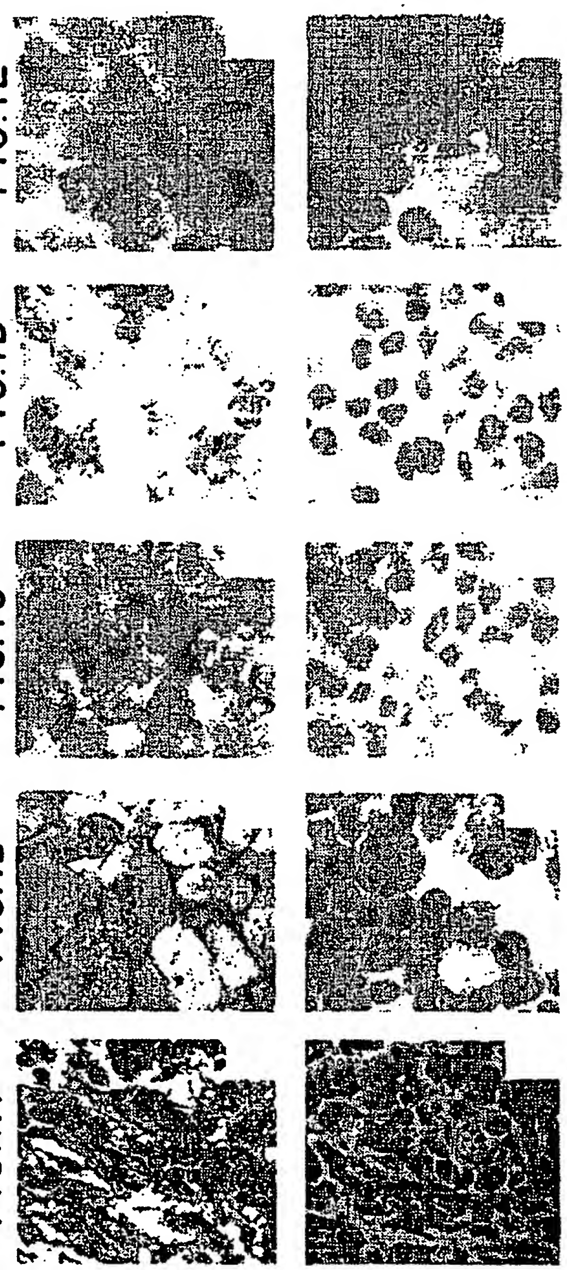


FIG.1A FIG.1B FIG.1C FIG.1D FIG.1E

FIG.1F FIG.1G FIG.1H FIG.1I FIG.1J

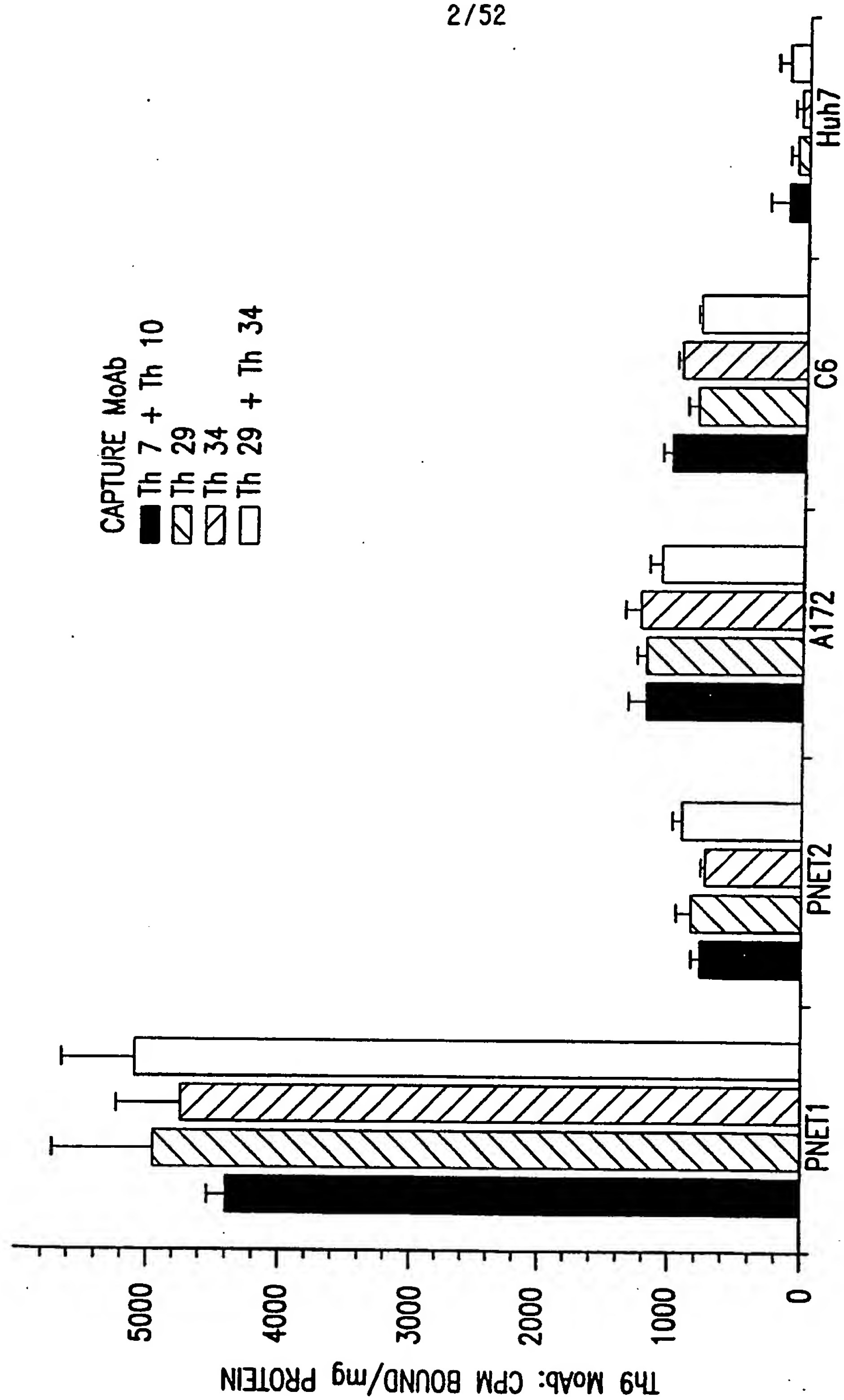

CAPTURE MoAb
Th 7 + Th 10
Th 29
Th 34
Th 29 + Th 34
Th9 MoAb: CPM BOUND/mg PROTEIN
0
1000
2000
3000
4000
5000
PNET1
PNET2
A172
C6
Huh7

FIG.2

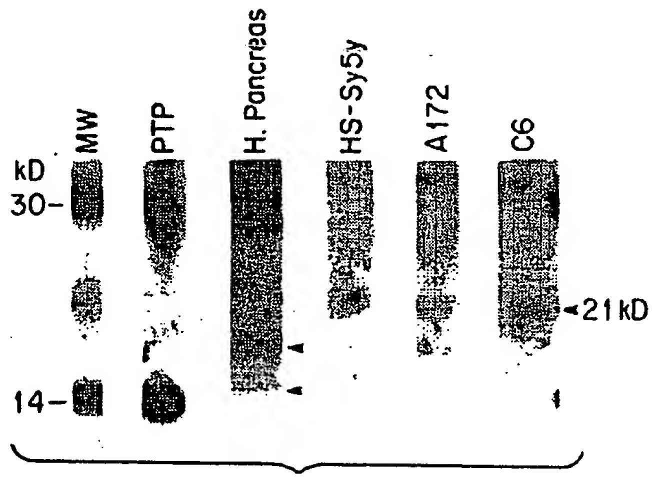


FIG. 3

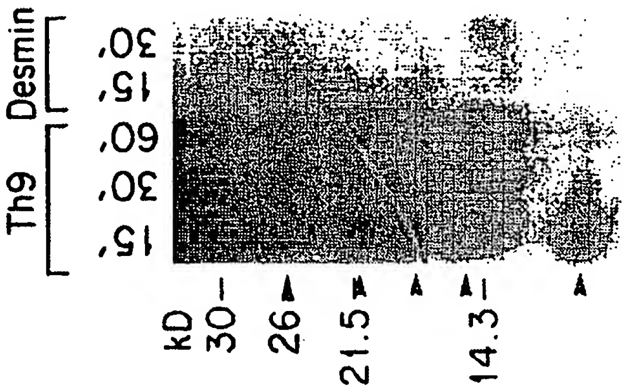

Th9
15'
30'
60'
Desmin
15'
30'
kD
30
26
21.5
14.3

FIG.4B

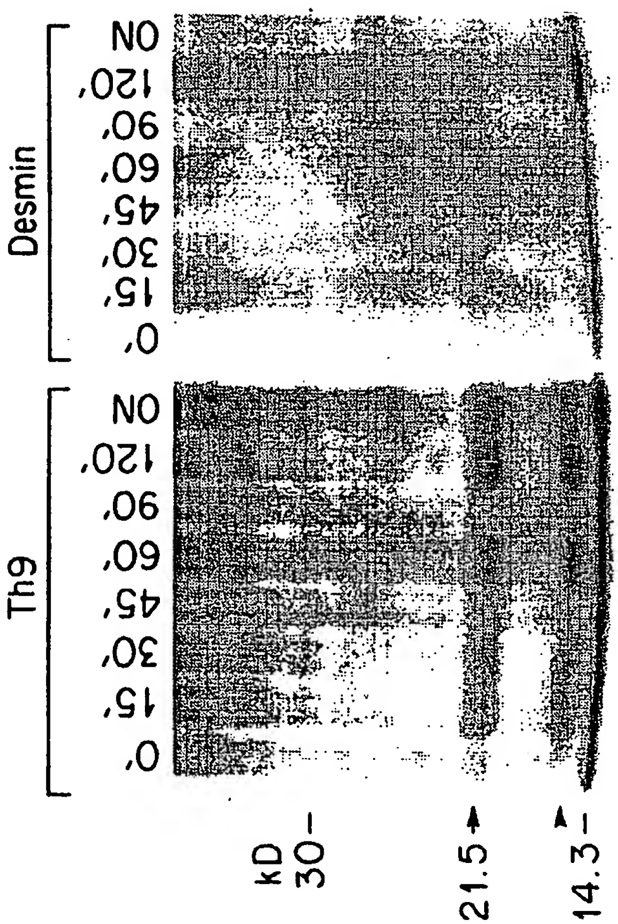

Th9
0'
15'
30'
45'
60'
90'
120'
NO
Desmin
0'
15'
30'
45'
60'
90'
120'
NO
kD
30
21.5
14.3

FIG.4A

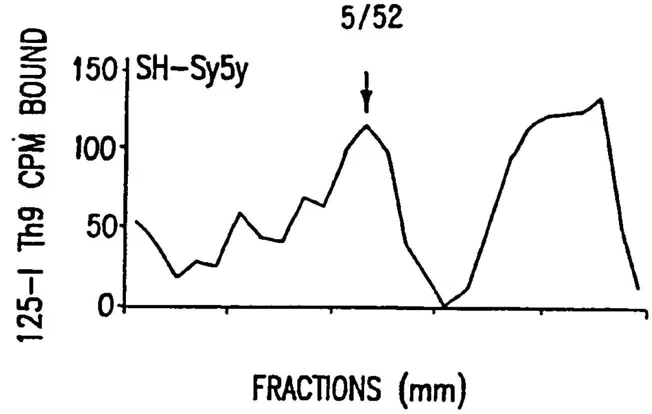


FIG.5A

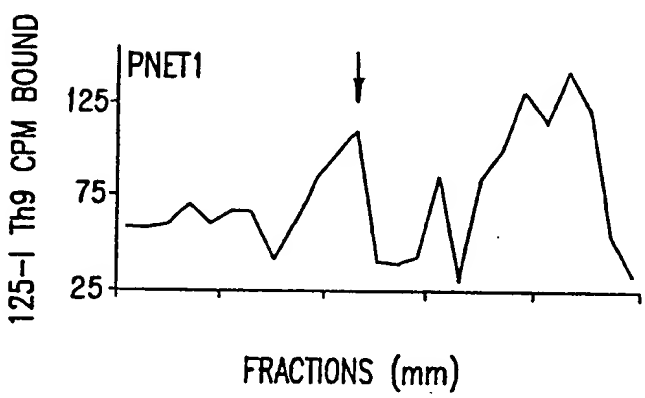


FIG.5B

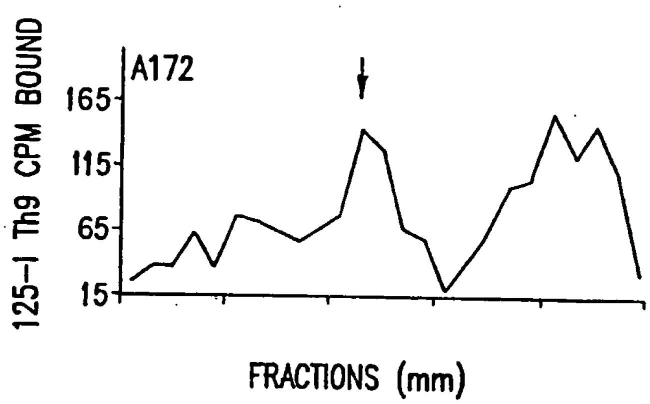
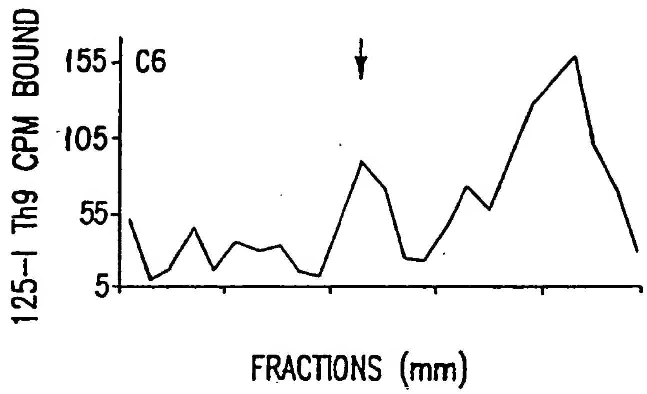
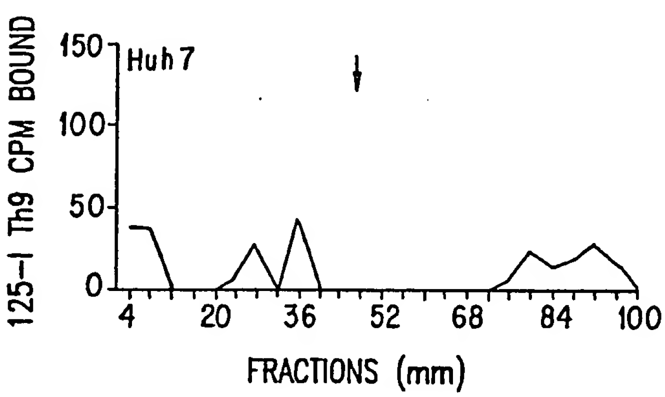


FIG.5C



155
105
55
5
C6
125-I Th9 CPM BOUND
FRACTIONS (mm)

FIG.5D



150
100
50
0
Huh 7
4
20
36
52
68
84
100
125-I Th9 CPM BOUND
FRACTIONS (mm)

FIG.5E

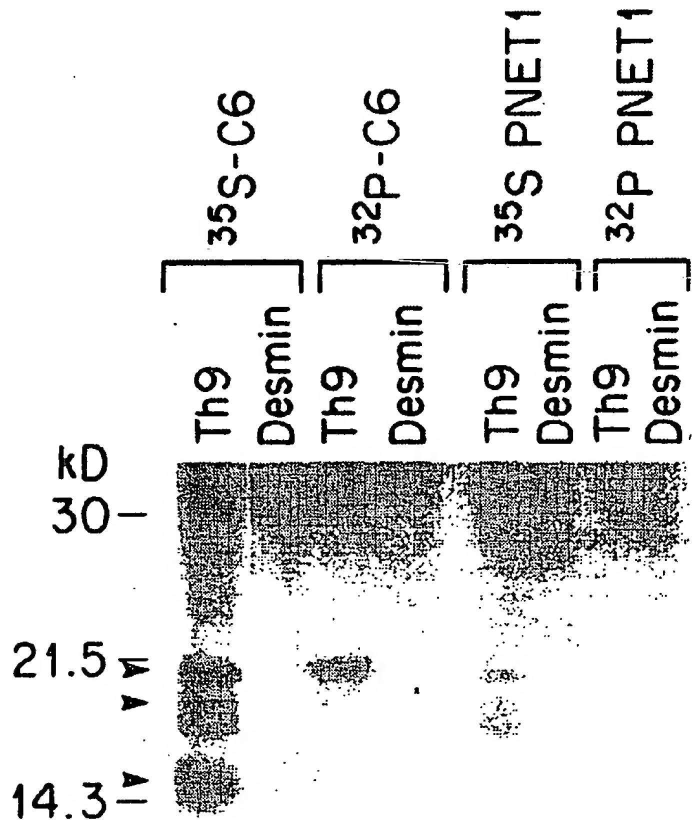

^{35}S-C6
^{32}P-C6
^{35}S PNET1
^{32}P PNET1
Th9
Desmin
Th9
Desmin
Th9
Desmin
Th9
Desmin
kD
30
21.5
14.3

FIG. 6

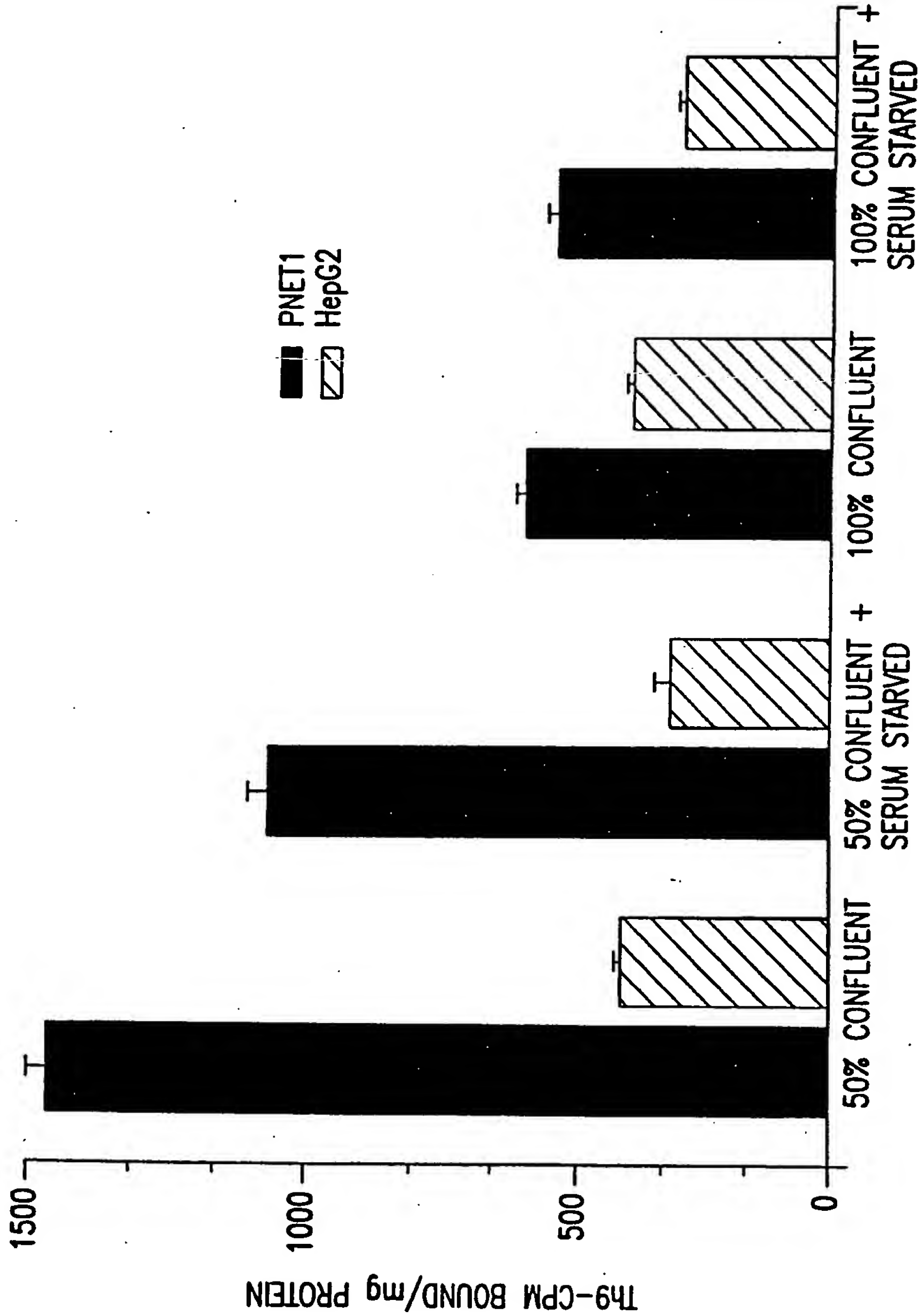

PNET1
HepG2
1500
1000
500
0
Th9-CPM BOUND/mg PROTEIN
50% CONFLUENT
50% CONFLUENT + SERUM STARVED
100% CONFLUENT
100% CONFLUENT + SERUM STARVED

FIG.7

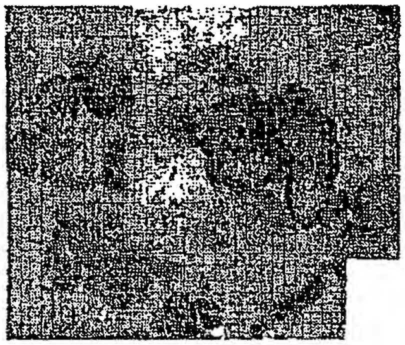


FIG. 8A

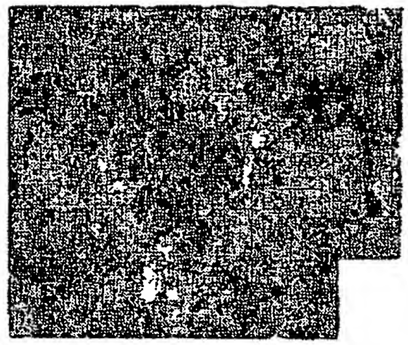


FIG. 8B

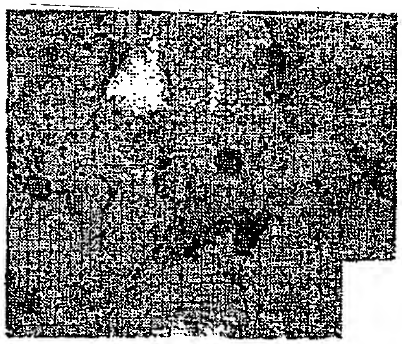


FIG. 8C

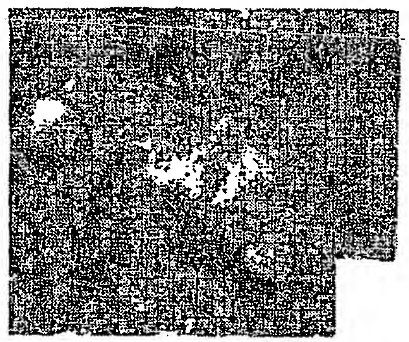


FIG. 8D

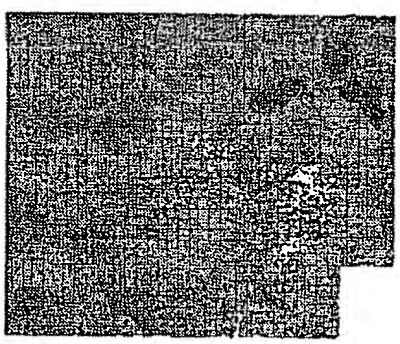


FIG. 8E

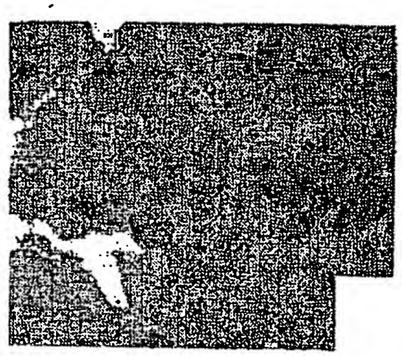


FIG. 8F

1-9a T7 SEQUENCE

Sequence Range: 1 to 1442

CGCTG CGCCC AGGCT GGCTC TGGAA AGCCT GTGCG GTCCT GGCAG GAAGC CCGGC CCGTG 60
GAGCA GGTTT TCGTT CTGCT TCAGC AATAA ATAAG GGTGA CCACA GGGAC TTTGC TTTTG 120
GTTTC CTTTC CTGTG AAAAG GTTGG TTTTA AAGTG AGATA CACTT TTCCG TAGAA CAAGT 180
GTTCT ATCTT TAAAA ACCCA AATTG CAGCA CCGTG GATTA CTGGT CTCAG AACAA CTCAT 240
TGCGC ATCAG ATTTG ACTCT CTGAT TTTCT GTCTA TTGGC CAAAT TGCCC TTTAA CTGCA 300
CCTGA ATCCT TTGTG TACTG ATGCC TTTGA GCTGG GCACC TTGGG AGAGT GTTGT GTTGC 360
TGTTT ACGGT TCTTC CTTCC CCTTG CTAAT TACAG TCTCT GGTGC CCAGC AAGCC CCTTT 420
GGCTT CCTTC CGTGA CTGGT CACGT TGTCT GCCTG GGCTC AGCGT GGACC TGCCC CATGC 480
TGCAG AACCT GGCCT CACCT GGACT TTTAC TAGAA TTGCC AGCTT CTCAA CTTAG CAGAT 540
CATCA CTCAT GCGGG CACAA GCAAA GATCA ACACT TTCTT TTTTG GTAAG CTTGA GTTTT 600
ACAAG TTATT TTTTG GTGAT GCGTA AGACA TTGCA GTGGG AAAGC ATTCA ACTTG AGTTT 660
ATTGG AGTTT GCTGT TGTAG CAGGT TTTAA CTCAG GAACA ACTCT TGTCT GATCT CTCGC 720
CCCTC TGCCG GGACT ACATT ACTGT CTCTC GGAGC CGGTA GCGTT GCTGT CGAGT CCCAG 780
GACTA TCTCT GCAGA CTGCT ATGCT CAGAT CGAAG TATTT CACAA GAATA CTTGT GTTTT 840
TAACA GCCCT TCCCC TGGAC GGTGC GCCAT GAGGG CCTCA TGTTA CGCAT TGCCT TTTCT 900
TTCTG TGGAT CCAGT ATCTT CCTCG GCTTT TTAGG GAGCA GGAAA AATGC GTCTG AGAGC 960
AACTC TTTTT AAAAA CCTGC CCTGT TGTAT ATAAC TGTGT CTGTT TCACC GTGTG ACCTC 1020
CAAGG GGGTG GGAAC TTGAT ATAAA CGTTT AAAGG GGCCA CGATT TGCCC GAGGG TTACT 1080
CCTTT GCTCT CACCT TGTAT GGATG AGGAG ATGAA GCCAT TTCTT ATCCT GTAGA TGTGA 1140
AGCAC TTTCA GTTTT CAGCG ATGTT GGAAT GTAGC ATCAG AAGCT CGTTC CTTCA CACTC 1200
AGTGG CGTCT GTGCT TGTCC ACATG CGCTG GGCGT CTGGA CCTTG AATGC CTGCC CTGGT 1260
TGTGT GGACT CCTTA ATGCC AATCA TTTCT TCACT TCTCT GGACA CCCAG GGCGC CTGTT 1320
GACAA GTGTG GAGAA ACTCC TAATT TAAAT GTCAC AGACA ATGTC CTAGT GTTGA CTACT 1380
ACAAT GTTGA TGCTA CACTG TTGTA ATTAT TAAAC TGATT ATTTT TCTTA TGTCA AAAAA 1440
AAA

FIG.9

WP5' SEQUENCE

Sequence Range: 1 to 313

GATCC CGTTT GACAG GTGTA CCGCC CCAGT CAAAC TCCCC ACCTG GCACT GTCCC CGGAG
CGGTC GCGCC CGCGC GACCA CGGAG CTCTG GGCGC CAGAA GCGAG AGCCC CTCGC TGCCC
CCCGC CTCAC CGGGT AGTGA AAAAA CGATG AGAGT AGTGG TATTT CACCG GCGGC CCGCG
AGGAC CCCCG CCCGA CCCAG TGCGG AACGG GGG

FIG.9A

```
9A•I-T7                                                    5    10    15    20
[ 386 ]   ---------------------------------------------CAcc GtgGa TtA-C tgGtC>
Human-PTP                                                  CATT GCACC TCAGC ATGGC

9A•I-T7     25    30    35    40    45    50   55    60    65    70    75
[ 386 ]   TCAGA aCAaC TCATt gcgCA T-CaG ATtTa CTctC TGAIt TTTCT GTCTa ttgGC CAttG>
Human-PTP TCAGA CCAGC TCATA CTTCA TGCTG ATCTC CTGCC TGATG TTTCT GTCTC AGAGC CAAGG

9A•I-T7   85    90    95    100   105   110   115         125   130    135   140
[ 386 ]   CCctt taaCt gcacC tGA-a TcCtt tgtGt aCtGA TCctt TGagC tG--G GCACC -tTG->
Human-PTP CCAAG AGGCC CAGAC AGAGT TGCCC CAGGC CCGGA TCAGC TGCCC AGAAG GCACC AATGC

9A•I-T7     145        150   155   160   165   170   175   180   185   190   195
[ 386 ]   ggAga G-T-g TtgTG tTgCT -gTTT AcgGt tctlC cT-tc cCCTt GcTaa tTaCA G-TCT>
Human-PTP CTATC GCTCC TACTG CTACT ACTTT AATGA AGACC GTGAG ACCTG GGTTG ATGCA GATCT

9A•I-T7     200  205    210   215   220   225   230   235   240   245   250   255
[ 386 ]   CTggT GCCAG cA-Ag ccccT ttGGC ttCCT tccGT gacTG gTCAC gttGt CtGcc tGgGC>
Human-PTP CTATT GCCAG AACAT GAATT CGGGC AACCT GGTGT CTGTG CT Q C CCAGG CCGAG GGTGC

9A•I-T7     260   270   275   280    285   290     300   305   310   315
[ 386 ]   CagcG TGGCC cCA-T GcTgc A-GAa ccTGG C-CTc AgGAC TTttc acT-a GaATT GcCCT>
Human-PTP CTTTG TGGCC TCACT GATTA AGGAG AGTGC CACTG ATGAC TTCAA TGTCT GGATT GGCCT

9A•I-T7   325   330   335   340   345   350   355   360   365   370   375
[ 386 ]   tCcTc A-aCt tAgcA GAtCa ttCaC Tcatg CgGCc aCA-a Gcaaa gaTca aCaCt TtC-t>
Human-PTP CCATG ACCCC AAAAA GAACC GCCGC TGGCA CTGGA GCAGT GGGTC CCTGG TCTCC TACAA

9a-t7                         10    15    20    25    30     35      40    45
[ 180 ]   -----------------TT tTCCT AgA-A cA-aG -gGtT cTatC T-TTA A--AA -Accc aaATT>
H REG GENE                 TT CTCCT ATAGA GATTG TTGAT TTGCC TCTTA AGCAA GAGAT TCATT

9a-t7         50   55  65    70    75    80    85    90    95  100   105   110
[ 180 ]   GCAGC aCcGC tgGtC TCAGA aCAaC TCATt gcgCA T-CaG ATtTa CTctC TGATt TTTCT>
H REG GENE GCAGC TCAGC ATGGC TCAGA CCAGC TCATA CTTCA TGCTG ATCTC CTGCC TGATG TTTCT

9a-t7       115   120   125   130   135   140   145   150   155
[ 180 ]   GTCTa tttGg CcAaa T-tGc cCT-T TTaaC tgCAc CtgAa TCTTT>
H REG GENE GTCTC AGAGC CAAGG TAAGA TCTCT TTTCC ACCAA CCAAC TCTTT
```

FIG.10

```
9A*1-T7                                             5    10    15    20    25    30
[ 130 ]     ----------------------------------------CAc cGtgG oTtA- CtgGt CTCAG AoCAo>
EXON2                                               CAT TGCAG CTCAG CATGG CTCAG ACCAG

9A*1-T7       35    40    45    50    55    60    65    70    75    80
[ 130 ]     CTCAT tgcgC AT-Co GATtT oCTct CTGAT tTTTC TGTCT ottgG CCAA>
EXON2       CTCAT ACTTC ATGCT GATCT CCTGC CTGAT GTTTC TGTCT CAGAG CCAA


H REG  GENE                5    10    15    20    25    30    35    40    45
[ 136 ]     ----------G oAtTc cTGgg cTCAo GtgAt CCTC- TColG coGTC TCC-- CA-oA gT-GC>
WP03-4 T7             G GAGTT TTGTC ATCAG GCCAG CCTCA TCCCG AGGTC TCCTC CACCA TTGGC


H REG GENE     50    55    60    65    70    75    80    85          95   100
[ 136 ]     tG-gG otgoC AGGcT -tGoG C-CAC C-AcA --ccA ggCCC oT-Co TCAGt ttotA TAAAG>
WP03-4 T7   CGTAG CCAGC AGGTT CAGTG CTCAC CGAAA GTAAA ATCCC CTCCT TCAGC AAGAA TAAAG

H REG GENE  105   110   115   120   125   130
[ 136 ]     oAAoA oAoAC CTTAo ooT-t gtTAg GcAA- ATA>
WP03-4 T7   CAATA TACAC CTTAG GTTCC ACTAA GTAAC ATA

WP03 8SP          120   115   110   105   100    95    90    85    80    75
[ 108 ]     -----<GTT-c TgTGo gTCTc AAttt gttcc TTCtT gGoAG CT--G tcTGG -TgA- AtCtG
EXON2             GTTGA TTTGC CTCTT AAGCA AGAGA TTCAT TGCAG CTCAG CATGG CTCAG ACCAG

WP03 8SP      70    65    60    55    50    45    40    35          30
[ 108 ]     <tTggT cCcTC tgtCT GcToT tCTGt CTG-T cTgTo TGTCT --G-t CCAtG
EXON2        CTCAT ACTTC ATGCT GATCT CCTGC CTGAT GTTTC TGTCT CAGAG CCAAG
```

FIG.10A

```
AD3-4-296                          260   255   250     245     240   235   230
[ 112 ]     ------------------<ACtA CcaAa C-CT- -GCat tA-AA --aAa tttCg gTtG- -GtCg
WP5'4/93                       ACCA CGGAG CTCTG GGCGC CAGAA GCGAG AGCCC CTCGC TGCCC

AD3-4-296   225    220   215   210   205   200   195    190   185   180   175   170
[ 112 ]      <aCCtC -ggAg C-aGa AcccA Acctc CGAgc AGtac A-TGc TAaga CttCa cCaGt CaaaG
WP5' 4/93     CCCGC CTCAC CGGGT AGTGA AAAAA CGATG AGAGT AGTGG TATTT CACCG GCGGC CCGCG

AD3-4-296       165   160   155   150     145   140   135   130   125   120   115
[ 112 ]     <-cGA- aCgta CtatA CtCAa T--tG AtCca ataAc TtGaC CaacG Gaaca AgTTA ccCTA
WP5' 4/93    AGGAC CCCCG CCCGA CCCAG TGCGG AACGG GGGAG TAGTC CCGGG GGCTC ACTTA TTCTA

AD3-4-296     105    100    95    90    85    80    75 70    65    60    55     50
[ 112 ]     <tAacA G-CgC Aatcc tAttC TAGAG TCcAt aTCAA CAGGG TtTaC gacCt CGaTG -TTgg
WP5' 4/93    CATTA GTCTC ACGTG CAGAC TAGAG TCAAG CTCAA CAGGG TCTTC TTTCC CGCTG ATTCC

AD3-4-296     45   40
[ 112 ]     <atCAg GaC
WP5' 4/93    GCCAA GTC

AD2 SP6F          10    15  20    25    30    35    40    45    50    55    60    65
[ 504 ]      AG-Tt TCaCT CTGTt gCCCA GGCTG gAGTG CAaTG GCaCA ATCct GGCTC ACTGC aAcCT>
1-9AT7-3 3   AGATC TCGCT CTGTC ACCCA GGCTG AAGTG CAGTG GCCCA ATCTC GGCTC ACTGC GAGCT

AD2 SP6F        70   75    80    85    90    95   100      105      110   115
[ 504 ]      CCgCC TCCCG aGcTC Aagca ATTCT CCTGC CTCA- -GCCT C-G-- --TGA GccGC TGGGA>
1-9AT7-3 3   CCACC TCCCG GGTTC ACTTC ATTCT CCTGC CTCAC TGCCT CAGCC TCTGA GTAGC TGGGA
```

FIG.10B

HB4-SEQ SEQUENCE

```
GAGGC GTATT ATACC ATGCT CCATC TGCCT ACGAC AAACA GACCT AAAAT CGCTC ATTGC   60
ATACT CTTCA ATCAG CCACA TAGCC CTCGT AGTAA CAGCC ATTCT CATCC AAACC CCCTG  120
AAGCT TCACC GGCGC AGTCA TTCTC ATAAT CGCCC ACGGG CTTAC ATCCT CATTA CTATT  180
CTGCC TAGCA AACTC AAACT ACGAA CGCAC TCACA GTCGC ATCAT AATCC TCTCT CAAGG  240
ACTTC AAACT CTACT CCCAC TAATA GCTTT TTGAT GACTT CTAGC AAGCC TCGCT AACCT  300
CGCCT TACCC CCCAC TATTA ACCTA CTGGG AGAAC TCTCT GTGCT AGTAA CCACG TTCTC  360
CTGAT CAAAT ATCAC TCTCC TACTT ACAGG ACTCA ACATA CTAGT CACAG CCCTA TACTC  420
CCTCT ACATA TTTAC CACAA CACAA TGGGG CTCAC TCACC CACCA CATTA ACAAC ATAAA  480
ACCCT CATTC ACACG AGAAA ACACC CTCAT GTTCA TACAC GTATC CCCCA TTCTC CTCCT  540
ATCCC TCAAC CCCGA CATCA TTACC GGGTT TTCCT CTTAA AAAAA AAAAA AAAA         590
```

HB4 PROTEIN

```
EAYYT MLHLP TTNRP KIAHC ILFNQ PHSPR SNSHS HPNPL KLHRR SHSHN RPRAY ILITI   60
LPSKL KLRTH SQSHH NPLSR TSNST PTNSF LMTSS KPR                             95
```

FIG.11A

15/52

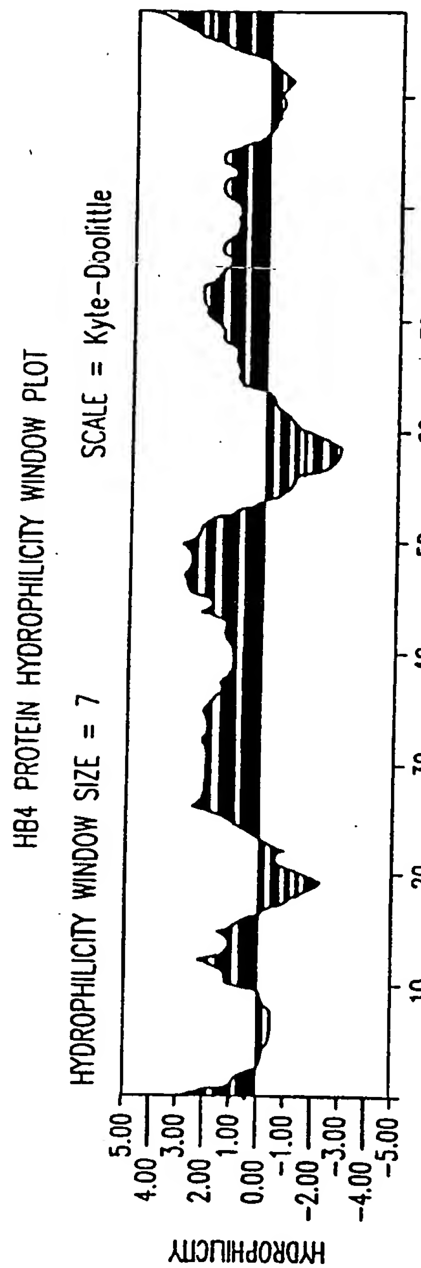


FIG.11B

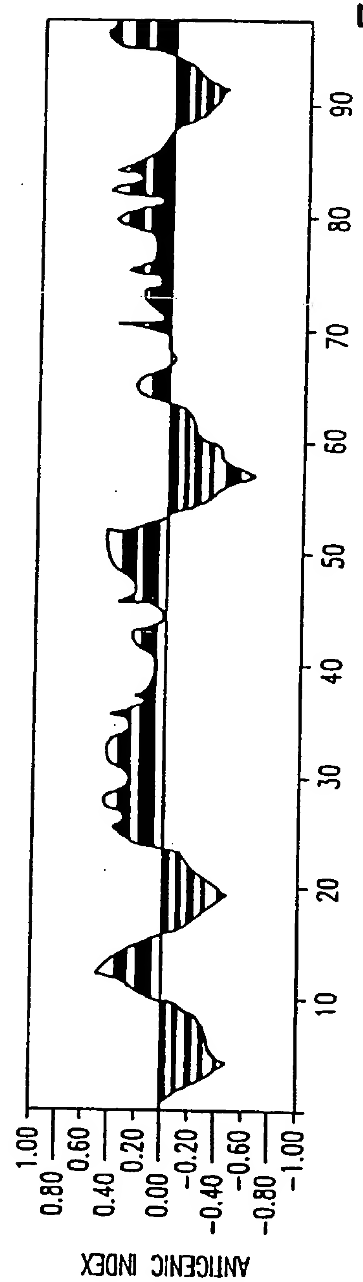


FIG.11C

```
HB4 SEQ                                                         ATaC>
[ 440 ]  ———————————————————————————————————————————————————————
Human-PTP                                                       ATGC

HB4-SEQ  15    20    25    30    35    40    45    50    55    60    65
[ 440 ]    C-AT- GCTCC atCTG C--CT ACgac AA-ac AGACC -T-A- AaaTc GcTca tTGCA tA-CT>
Human-PTP  CTATC GCTCC TACTG CTACT ACTTT AATGA AGACC GTGAG ACCTG GGTTG ATGCA GATCT

HB4-SEQ     70    75    80    85    90    95   100   105   110   115   120
[ 440 ]    CTtca atCAG cACAT -Agcc CtcG- tAgta acaG- CcaTt CTCAt CCAaa CCccc tGaag>
Human-PTP  CTATT GCCAG AACAT GAATT CGGGC AACCT GGTGT CTGTG CTCAC CCAGG CCGAG GGTGC

HB4-SEQ  125   130   135   140   145   150   155   160   165   170   175
[ 440 ]    CTTca ccGgC gCAgT cATT- ctcAt AaTcG C-Cca cgGgC TTacA T-cCT -cATT actaT>
Human-PTP  CTTTG TGGCC TCACT GATTA AGGAG AGTGG CACTG ATGAC TTCAA TGTCT GGATT GGCCT

HB4-SEQ  180   185   190   195   200   205   210   215   220   225 230   235
[ 440 ]    tC-TG cCaqC AAAct cAAaC taCGa acGCA CT-cA -CAGT cGcat CaTaa TCTCt ctCAA>
Human-PTP  CCATG ACCCC AAAAA GAACC GCCGC TGGCA CTGGA GCAGT GGGTC CCTGG TCTCC TACAA

HB4-SEQ  240   245   250   255   265 270 275   280   285   290   295   300   305
[ 440 ]    GgaCT -tcaa AcTct ActCC CAAGC ttTGT gAcTt CTaGC aACct cGctA aCCTc gCCTt>
Human-PTP  GTCCT GGGGC ATTGG AGCCC CAAGC AGTGT TAATC CTGGC TACTG TGTGA GCCTG ACCTC

HB4-SEQ  310   315   320   325   330   340   345   350   355   360365   370
[ 440 ]    AccCc CActA TTaac ctAcT GGgAG aATGT G-CTa GT-AA -cCAc GTTCT CCTTc aaaTa>
Human-PTP  AAGCA CAGGA TTCCA GAAAT GGAAG GATGT GCCTT GTGAA GACAA GTTCT ccTTT GTCTG

HB4-SEQ        375   380   385   390   395   400   405   410   415   420   425
[ 440 ]    tcAcT ctcct ActTA cAGG- A-CT- cAAcA TACta GTCcA GccCT -ATaC tcCct cTACA>
Human-PTP  CAAGT TCAAA AACTA GAGGC AGCTG GAAAA TACAT GTCTA GAACT GATCC AGCAA TTACA

HB4-SEQ  430   435   440   445   450   455   460   465   470 475   480   485
[ 440 ]    tattt acCAc AAcac AAtgg GGctC A-CTC aCCcA C-CAc aTtAA CCata AaACc CTCaT>
Human-PTP  ACGGA GTCAA AAATT AAACC GGACC ATCTC TCCAA CTCAA CTCAA CCTGG ACACT CTCTT

HB4-SEQ  490   495   500   505   510   515   520   525   530   540   545
[ 440 ]    -TCac acGAG -aaaa Caccc TcATg TTC-A TAcac cTA-- TcCCC CAtTC TTcct AtCCc>
Human-PTP  CTCTG CTGAG TTTGC CTTGT TAATC TTCAA TAGTT TTACC TACCC CAGTC TTTGG AACCT

HB4-SEQ      550   555   560 565   570   575   580   585   590
[ 440 ]    TcAAc cccgA cAtcA AcCgg GTTTC CtCTt AAAAA AAAAA AAAAA A>
Human-PTP  TAAAT AATAA AAATA AACAT GTTTC CACTA AAAAA AAAAA AAAAA A
```

FIG.11D

```
H REG GENE      2260  2265  2270  2275  2280  2285  2290   2295  2300  2305
[ 284 ]      ——C tTcTT -TttC AgGC- CoAgo gGCCc A-GAC AgAgt tgCC- ccAgg CcCgg ATcag>
HB4-SEQ        C GTATT ATACC ATGCT CCATC TGCCT ACGAC AAACA GACCT AAAAT CGCTC ATTGC

H REG GEN2310  2315  2320  2322330  2335  2340   2345  2350  2355  2360  2365  2370
[ 284 ]      cTgCc CagoA ggCAc CoACc TAtCg CTCcT AcT-g CtoCt AcTtT oATgo AgACC gCgoC>
HB4-SEQ      ATACT CTTCA ATCAG CCACA TAGCC CTCGT AGTAA CAGCC ATTCT CATCC AAACC CCCTG

H REG GBNE      2375  2380  23852390  2395  2400 2405  2410  2415  2420  2425   2430
[ 284 ]      -AcCT gggtt GotGC AGTgt gagTg AggAg oGCgt gtGCG aaggg AgoCT CATgA -oggg>
HB4-SEQ      AAGCT T QCC GGCGC AGTCA TTCTC ATAAT CGCCC ACGGG CTTAC ATCCT CATTA CTATT

H REG GENE     2435  2440   2445   2450  2455  2460  2465 2470  2475   2480  2485
[ 284 ]      agGgg aACC- tgC-C ActCT -CcAg tGtgt TCAgt GgCGC Aatgo gAT-g agoCT gAAcc>
HB4-SEQ      CTGCC TAGCA AACTC AAACT ACGAA CGCAC TCACA GTCGC ATCAT AATCC TCTCT CAAGC

H REG GENE 24902495  2500  2505  2510   2515  2520    2525    2530   2535  2540
[ 284 ]      cCTTt AtACT oTcoT CagcC ccA-A oCTTT ccoAT —CTo CT—t tAtCC -CotT AttCo>
HB4-SEQ      ACTTC AAACT CTACT CCCAC TAATA GCTTT TTGAT GACTT CTAGC AAGCC TCGCT AACCT

H REG GENE2545  2550  2555  2560  2565  2570     2580  2585    2590  2595  2600
[ 284 ]      gcoCo TtCCC ogCAC oAagA ACCTg gTGGG tG-AC agcoT cotC- AcggA Cotto cTCTg>
HB4-SEQ      CGCCT TACCC CCCAC TATTA ACCTA CTGGG AGAAC TCTCT GTGCT AGTAA CCACG TTCTC

H REG GEN2605   2610  2612620 2625   2630  2635  2640  2645  2650  2655  2665  2670
[ 284 ]      CTG-T CcttT tTCAC cCTCC T-CTT ggAGG ACTCA gtATA tccGT CACAo CCCTc cACTg>
HB4-SEQ      CTGAT CAAAT ATCAC TCTCC TACTT ACAGG ACTCA ACATA CTAGT CACAG CCCTA TACTC

H REG GENE  2675  2680     2685    2690    2695  2700    2705  2710  2715   2720
[ 284 ]      ogTCT cCAT- TTT-C ttC—- tgCAA co—G CTCto T-tgC CAgoA CATgA A-ttC gggcA>
HB4-SEQ      CCTCT ACATA TTTAC CACAA CACAA TGGGG CTCAC TCACC CACCA CATTA ACAAC ATAAA

H REG GENE 2725   2730   2735    2740
[ 284 ]      A-CCT -ggTg tC-tG tG—c tCACC C>
HB4-SEQ      ACCCT CATTC ACACG AGAAA ACACC C
```

FIG.11E

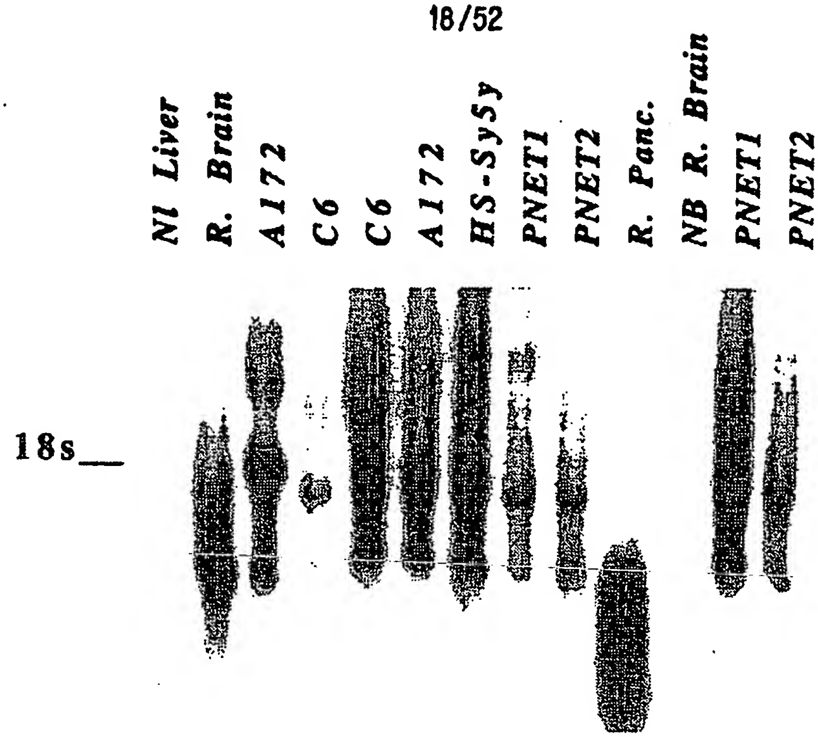


FIG.12A

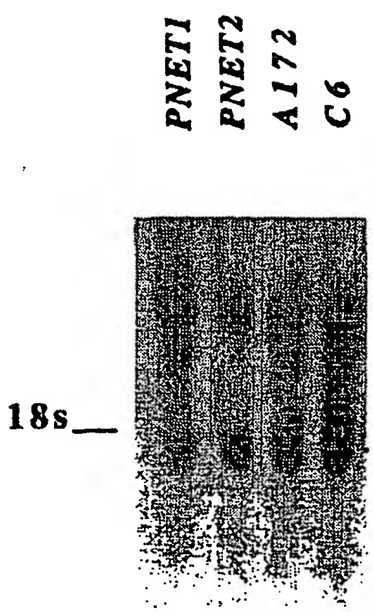


FIG.12B

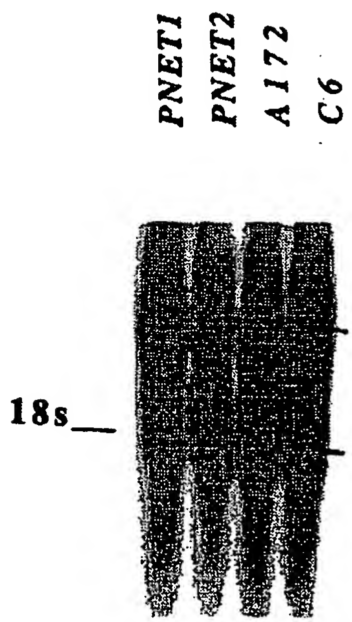


FIG.12C

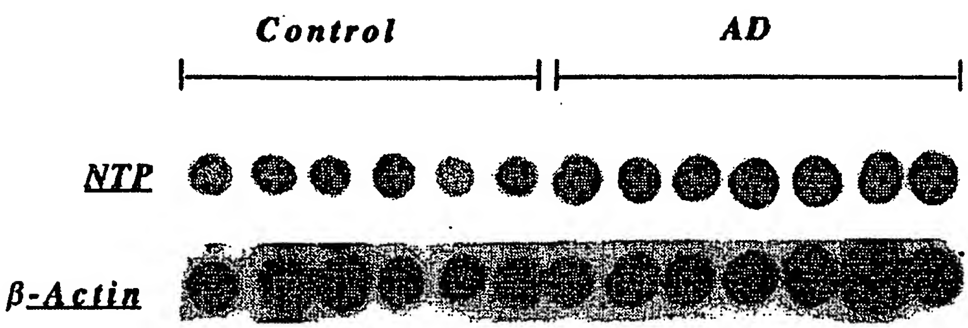


FIG. 13A

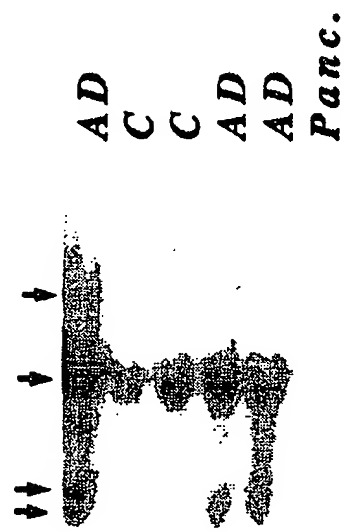


FIG.13B

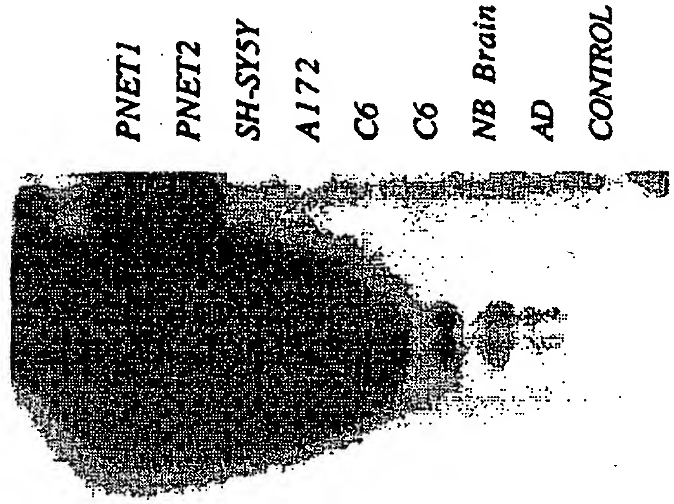


FIG. 14A

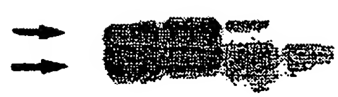


FIG. 14B

FIG. 14C

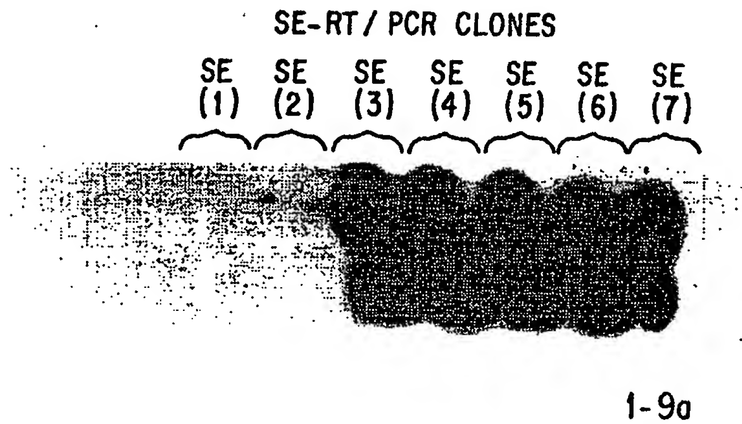


FIG. 15A

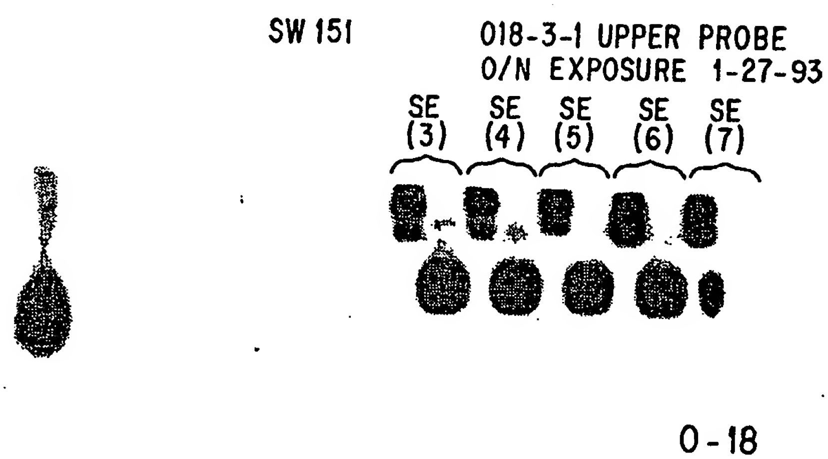


FIG. 15B

AD2-2 T7

GTTCT TAGTC TATCT CTTGT ACAAA CGATG TGCTT TGAAG ATGTT AGTGT ATAAC AATTG 60
ATGTT TGTTT TCTGT TTGAT TTTAA ACAGA GAAAA AATAA AAGGG GGTAA TAGCT CCTTT 120
TTTCT TCTTT CTTTT TTTTT TTCAT TTCAA AATTG CTGCC AGTGT TTTCA ATGTA GGACA 180
ACAGA GGGAT ATGCT GTAGA GTGTT TTTAT TGCCT AGTTG ACAAA GCTGC TTTTG AATGC 240
TGGTG GTTCT ATTCC TTTGC ACATC ACGAC ATTTT ATAAT CATAG TTAAA TCGTA TATGA 300
CAAAA ATGCT CTGAT CTGAT GCCAA AGGTC AATTC AGTGT ATATA ACCTG AACAC ACTCA 360
TCCAT TGCGT TT 372

AD2-2 T7 PEP

MFVFC LILNR EKIKG GNSSF FLLSF FFSFQ NCCQC FQCRT TEGYA VECFY CLVDK AAFEC 60
WWFYS FDT

FIG.16A

AD2 SP6F

ACTGT CTCCC CCTTT GATAG GGACA CTAAA GTGGT CTGTA CTTGG GTAGA GGATG GCANG 60
TTAAG AATTA AAATC GTCTG GGTGC GGTCT GCACG CTTGT AATCC CAGCA CTTTG GGAGG 120
CTGAG GCGGG CGGAT CACCT GAGGT CAGGA GTTCG ACACC AGCCT GATGA ACATG GAGAA 180
ACCCC ATCTC TACTA AAAAT ACAAA TATTA GCTGG GCGTT GTCGC GCGCC TGTAA TCCCA 240
GCGGC TCACG AGGCT GAGGC AGGAG AATTG CTTGA GCTCG GGATG GCGGA GGTTG CAGTG 300
AGCCA GGATT GTGCC ATTGC ACTCC AGCCT GGGCA ACAAG AGTGA AACTC TGTCT CAAAA 360
AAAAA AAAAA AAAAA AA 377

FIG.16D

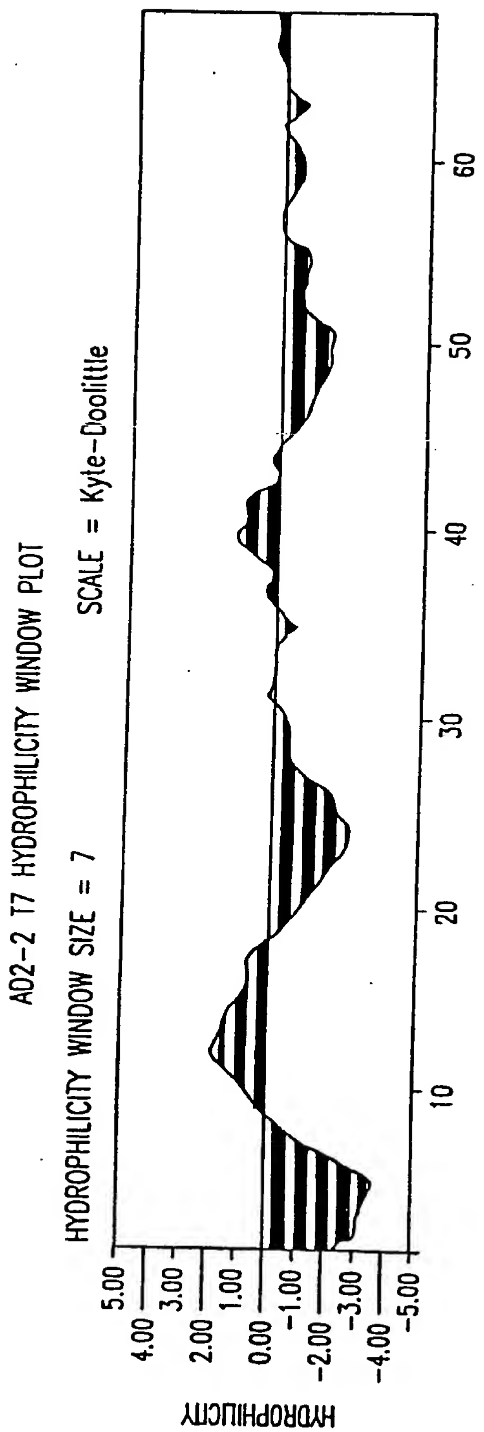


FIG.16B

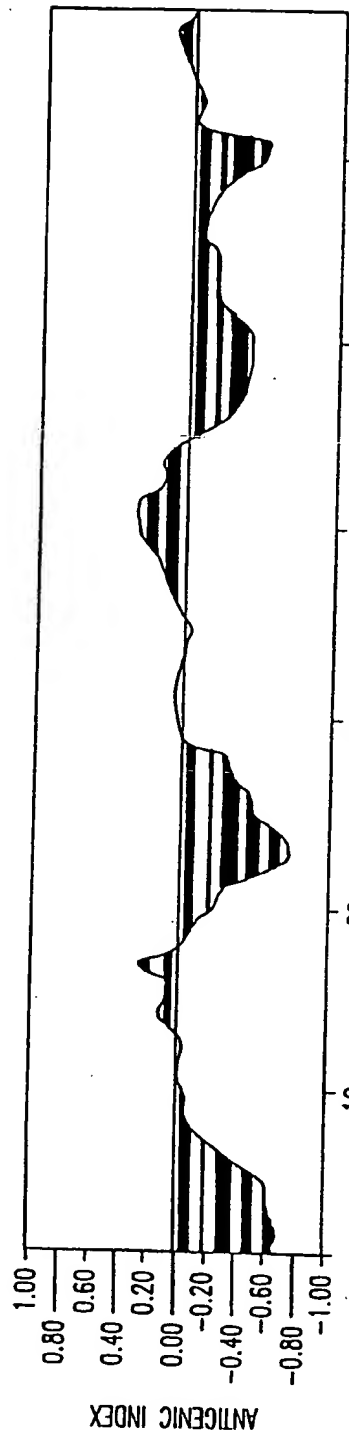


FIG.16C

AD2-2 SEQUENCE

```
GCGTAAACAC ATTTTTGTTC TTAGTCTATC TCTTGTACAA ACGATGTGCT TTGAAGATGT   60
TAGTGTATAA CAATTGATGT TTGTTTTCTG TTTGATTTTA AACAGAGAAA AAATAAAAGG  120
GGGTAATAGC TCCTTTTTTC TTCTTTCTTT GATTTTAAAC AGAGAAAAAA TAAAAGGGGG  180
TAATAGCTCC TTTTTTCTTC TTTCTTTTTT TTTTTTCATT TCAAAATTGC TGCCAGTGTT  240
TTCAATGATG GACAACAGAG GGATATGCTG TAGAGTGTTT TATTGCCTAG TTGACAAAGC  300
TGCTTTGAAT GCTGGTGGTT CTATTCCTTT GACACTACGC ACTTTTATAA TACATGTTAA  360
TGCTATAGGA CAAGATGCTC TGATTCCTGA GTGCCAGAGG TTCAATTCAG TGTATATAAC  420
TGAACACACT CATCCATTTG TGCTTTTGTT TTTTTTATGG TGGCTTAAAG GTAAAGAGCC  480
CATCCTTTGC AAGTCATCCA TGTTGTTACT TAGGCATTTT ATCTTGGCTC AAATTGTTGG  540
AAGAATGGTG GCTTGTTTCA TGGTTTTTGT ATTTGTGTCT AATGCACGTT TTAACATGAT  600
AGACGCAATG CATTGTGTAG CTAGTTTTCT GGAAAAGTCA ACTCTTTTAG GAATTGTTTT  660
TCAGATCTTC AATAAATTTT TTCTTTAAAT TTCAAAGAAC AATGTGCTTG TGTTGATGCC  720
TTACAAAAAC CATTGTATAT TTGTGTATTC CTTCTTGTAT TTAGAGAGTG GTTTTTCAGG  780
TGCGTGCTTT GTTTTCTGGT ATGGCCTTTA TGGAATGAGA CGCTTTAGCT TTGGTACGTA  840
GCGCTAATCC ATAGCAGCTT TGGCAGTTTC GTGTCTTGAG TCTTAGCTAA AAAGTTAGAA  900
GTTTACATGA CTGTTTTTTT TATTTTCCCT AAATTATTAC TTACTCTGAG CATTAATTAA  960
GGGCATTTTC ACCTGTGTAA AATTATGGTC AGCTTTTTTC TGTCTATAAT TGTTTACTTT 1020
TGTGGGTTTA CTCTAGAAAC ATGAGCCAAA AATGTCAATA GACAACACAG TATTAAAATA 1080
ACCCAAAAGT TGTAAAGGGC AACGTTTCTC CCCTTTGATA GGGACACTAA AGTGGTCTGT 1140
ACTTGGGTAG AGGATGGCAG ACGTTAAGAA TTAAAATGCG TCTGGGTGCG GTCTCACGCT 1200
TGTAATCCCA GCACTTTGGG AGGCTGAGGC GGGCGGATCA CCTGAGGTCA GGAGTTCGAC 1260
ACCAGCCTGA TGAACATGGA GAAACCCCAT CTCTACTAAA AATACAAATA TTAGCTGGGC 1320
GTTGTCGCGC GCCTGTAATC CCAGCGGCTC ACGAGGCTGA GGCAGGAGAA TTGCTTGAGC 1380
TCGGGATGCC GGAGGTTGCA GTGAGCCAGG ATTGTGCCAT TGCACTCCAG CCTGGGCAAC 1440
AAGAGTGAAA CTCTGTCTCA AAAAAAAAAA AAAAAAAAAA                      1480
```

FIG.16E

AD3-4 SEQUENCE

ttgggtgaggtgga

ATGAT	GGTCT	GTTGG	AATCG	GTTTG	GTAAA	TGGGT	TTATT	TCATA	TCCGC	TATCT	TTAAC	60
TTTGG	ACCGC	GTTAT	CTATA	TCATG	GCGTT	CCTTT	CTACT	TTTTA	ATATT	GGTTC	GTATT	120
ATATC	GTTCC	TGATT	GGGGA	TATGG	AAGAC	GTATT	ACTTA	ATTGT	ACTTT	ATTGA	AACGT	180
TCCTC	TCGGT	TTCGA	TTCTG	GGGGC	TTTGG	TCTGC	TCGAT	GGATT	CTTGT	CGATT	TTCTC	240
GTGTG	GCAGT	AACAT	ACCGT	TTTAT	CACCC	TTCTA	AATAT	CCCAT	CTCCC	GCTGT	TTGGT	300
AGGCT	CGGAA	CACTA	TCGAC	CAACA	GGTTC	TATCT	AGAAT	CAAGT	TGGAA	ATTAA	ACGGT	360
GTCTT	GG											367

AD3-4 PROTEIN

MMVCW	NRFGK	WYYFI	SAIFN	FGPRY	LYHGV	PFYFL	ILVRI	ISFLI	GDMED	VLLNC	TLLKR	60
SSRFR	FWGAL	VCSMD	SCRFS	RVAVT	YRFIT	LLNIP	SPAVW	MARNT	IDQQV	LSRIK	LEIKR	120
CL												122

FIG.16F

AD3-4T7

CCCAC	AGGTC	CTAAA	CTACC	AAACC	TGCAT	TAAAA	AATTT	CGGTT	GGTCG	ACCTC	GGAGC	1180
AGAAC	CCAAC	CTCCG	AGCAG	TACAT	GCTAA	GACTT	CACCA	GTCAA	AGCGA	ACGTA	CTATA	1240
CTCAA	TTGAT	CCAAT	AACTT	GACCA	ACGGA	ACAAG	TTACC	CTAGG	GATAA	CAGCG	CAATC	1300
CTATT	CTAGA	GTCCA	TATCA	ACAAT	AGGGT	TTACG	ACCTC	GATGT	TGGAT	CAGGA	CATCC	1360
CGATG	GTGCA	GCCGC	TATTA	AAGGT	TCGTT	TGTTC	AAACG	ATTAA	AGTCC	TCGTG	TCTGA	1420
GTTCA	GACCG	AAGTA	ATCCA	GGTCG	GTTTC	TATCT	TCTTC	AAATT	CCTCC	CTGTA	CCGAA	1480
AGGAC	TAATG	AGAAA	TAAGG	CCTAC	TTCAC	AAAGC	GGCCT	TCCCC	CGTAA	TGATA	TCATC	1540
TCAAC	TTAGT	ATTAT	ACCCA	CACCC	ACCCA	AGAAC	AGGGT	TTGTT	AAAAA	AAAAA	AAAAA	1600

FIG.16I

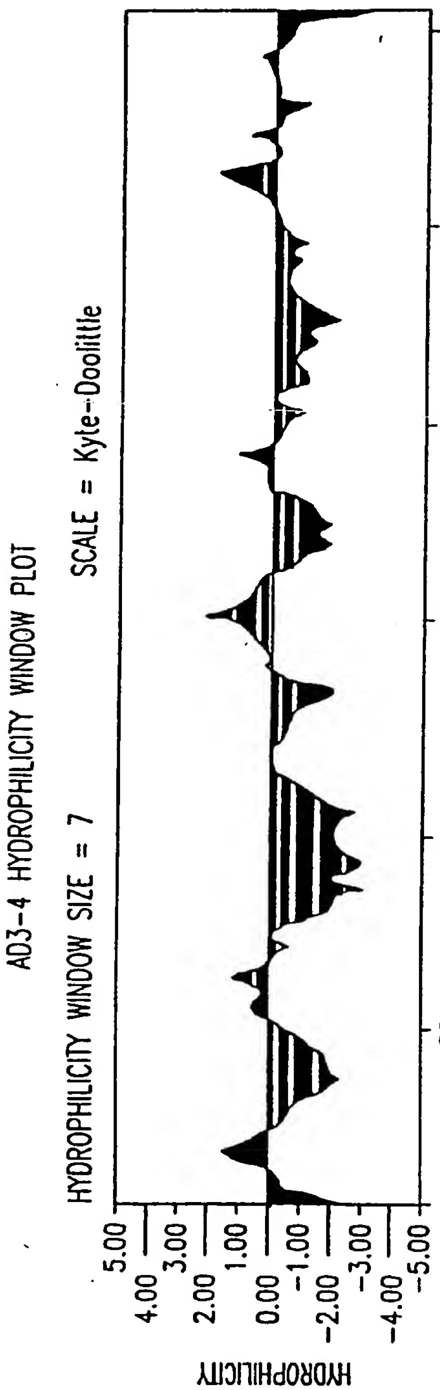


FIG.16G

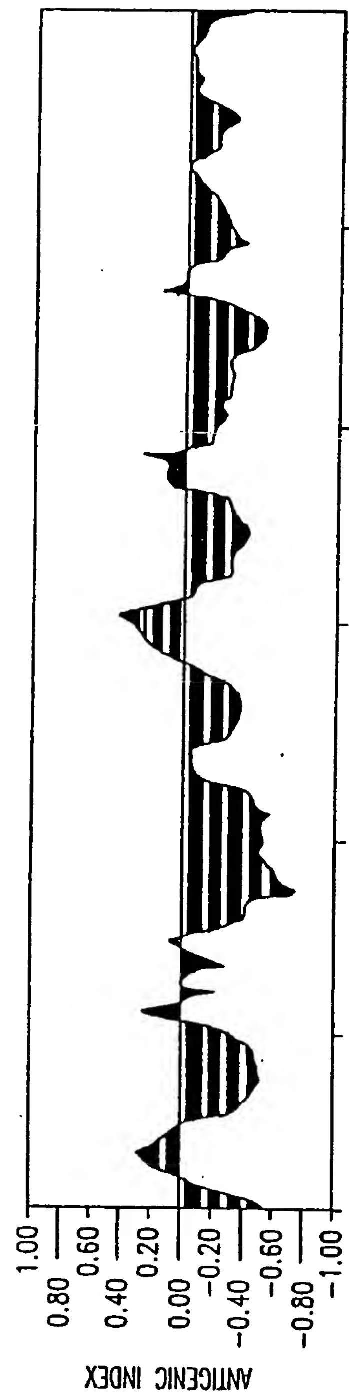


FIG.16H

AD3-4SP SEQUENCE

```
AACCCACTCC ACCTTACTAC CAGACAACCT TAGCCAAACC ATTTACCCAA ATAAAGTATA   60
GGCGATAGAA ATTGAAACCT GGCGCAATAG ATATAGTACC GCAAGGAAAG ATGAAAAATT  120
ATAACCAAGC ATAATATAGC AAGGACTAAC CCCTATACCT TCTGCATAAT GAATTAACAT  180
GAAATAACTT TGCAAGGAGA GCCAAAGCTA AGACCCCCGA AACCAGACGA GCTACCTAAG  240
AACAGCTAAA AGAGCACACC GTCATTGTAT GGCAAAATAG TGGGAAGATT TATAGGGTAG  300
AGGCGACAA ACCATCCGAG CCTTGTGATA GCTGGTTGTC CAAGATAGAT CTTAGTTCAA   360
CCTTTAATTT GCCACAGAAC C                                             381
```

FIG.16J

AD3-4T7 SEQUENCE

```
TTTTTTTTTT TTTTTAACAA ACCCTGTTCT TGGGTGGGTG TGGGTATAAT ACTAAGTTGA   60
GATGATATCA TTACGGGGGA AGGCCGCTTT GTGAAGTAGG CCTTATTTCT CATTAGTCCT  120
TTCGGTACAG GGAGGAATTT GAAGAAGATA GAAACCGACC TGGATTACTT CGGTCTGAAC  180
TCAGACACGA GGACTTTAAT CGTTTGAACA AACGAACCTT TAATAGCGGC TGCACCATCG  240
GGATGTCCTG ATCCAACATC GAGGTCGTAA ACCCTATTGT TGATATGGAC TCTAGAATAG  300
GATTGCGCTG TTATCCCTAG GGTAACTTGT TCCGTTGGTC AAGTTATTGG ATCAATTGAG  360
TTTAGTAGTC CGCTTGGAGT GGTGAAGTCT AGAATGTCCT GTTCGGGGGT TGGTTTCTGC  420
TCCCAGGTCG CCCCAACCGA ATTTTTTATT GAAGGTTGGG TAGTTTAGCA CCTGTGGGTT  480
GGTAAGGTAC TGTTGGAATT AATAAATTAA AGCTCCATAG GGTCTCCTCG TCTTGTTGTG  540
TAATGCCCCC CTCTCCACGG GAAGGTCAAT TCCACTGGTT AAAAGTAAGA GAAAGCTGAA  600
CCCTCGGGGA GCCATCCATA CAGGTCCCC                                    629
```

FIG.16K

AD4-4 SP6 SEQUENCE

Sequence Range: 1 to 256

```
GCGGG TAAAT TGGTT TGTTA TTTTT TAAAA AAAAC TTGCA TGTTT AAAAA AAAGT TGATT  60
GCTTC AAATT TCTGC TACTA ACTTC AAGCT ATGGG AGTTT GGCAG TAGTC ACTTG AGGAT 120
TTTTT TTCCA ATTCT TTTCT TTTTG TTGTT AAAGC TGTAC TTCAG TGAAC AGAAA AATTG 180
CCAAG CAAAC TAATG GACTA TAAAG CGTAA TTTGA CTGTG TGGGA CTAAA CTACA GAGCC 240
TACTT GACCA GTGGA T                                                     256
```

FIG.16L

AD4-4 T7F SEQUENCE

Sequence Range: 1 to 270

```
CATGT TTAAA AAAAA GTTGA TTGCT TCAAA TTACT GCTAC TAACT TCAAG CTATG GGAGT  60
TTGGC AGTAG TCACT TGAGG ATTTT TTTTC CAATT CGTTT TCATT TTTGT TGTTA AAGCT 120
CGTAC TTCAG TGAGA CAGAA AAATT GCCAA GCTAA ACTAA TGGTC TATAA AAGCG TAATT 180
TGCAT GTGTG GGCAA AAACT ACAGA GCCTC AATTG CCACT GAGGT ATAGT ACAAA GTTTT 240
AATAC ATTTT GTAAA TCAAA TTGAA AGAAA                                   270
```

FIG.16M

AD4-4 SEQUENCE

```
CATGTTTAAA AAAAAGTTGA TTGCTTCAAA TTACTGCTAC TAACTTCAAG CTATGGGAGT  60
TTGGCAGTAG TCACTTGAGG ATTTTTTTTC CAATTCGTTT TCATTTTTGT TGTTAAAGCT 120
CGTACTTCAG TGAGACAGAA AAATTGCCAA GCTAAACTAA TGGTCTATAA AAGCGTAATT 180
TGCATGTGTG GGCAAAAACT ACAGAGCCTC AATTGCCACT GAGGTATAGT ACAAAGTTTT 240
AATACATTTT GTAAATCAAA TTGAAAGAAA                                  270
```

FIG.16N

AD16c-T7 SEQUENCE

```
TCTGC CCAGG CTGGT CTGAA ATTCC TGGGC TGAAG TGATC CTCCA GTCTT GGCCT CCCAA  60
AGTGC TGGGA TTACA GGCAT GAGCT ACTGA GCCTA GCCTT AATGA TTAAT TTTAG AGTGA 120
TGGCT TGTAC CTTCA AGACA CATAT AGATT GAGAC AGAAA ATTTC CATCG TCCCC GAGAA 180
AACT                                                      184
```

AD16c-T7 PEP

```
          5     10    15    20    25    30    35    40    45    50    55    60
SSSLG LPKCW DYRHE LLSLA LMINF RVMAC TFKQH IELRQ KISIV PRKLC CMCPV CPVKI

   65    70    75
ALLTI NGHCT WLPAS
```

FIG.16O

SUBSTITUTE SHEET (RULE 26)

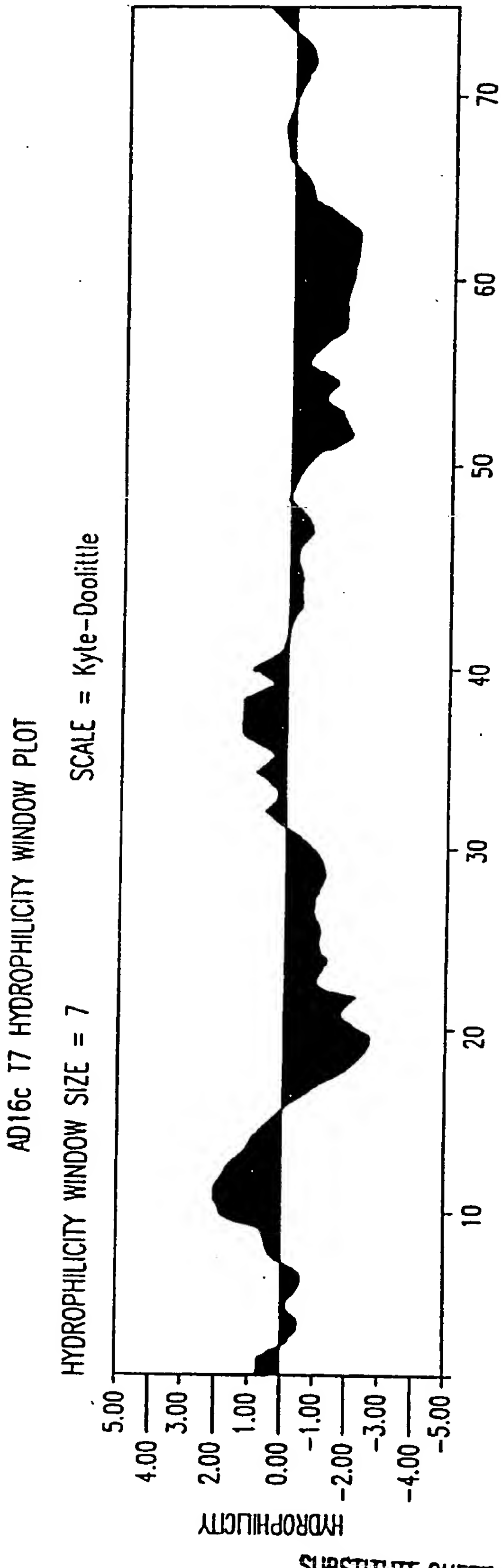


FIG.16P

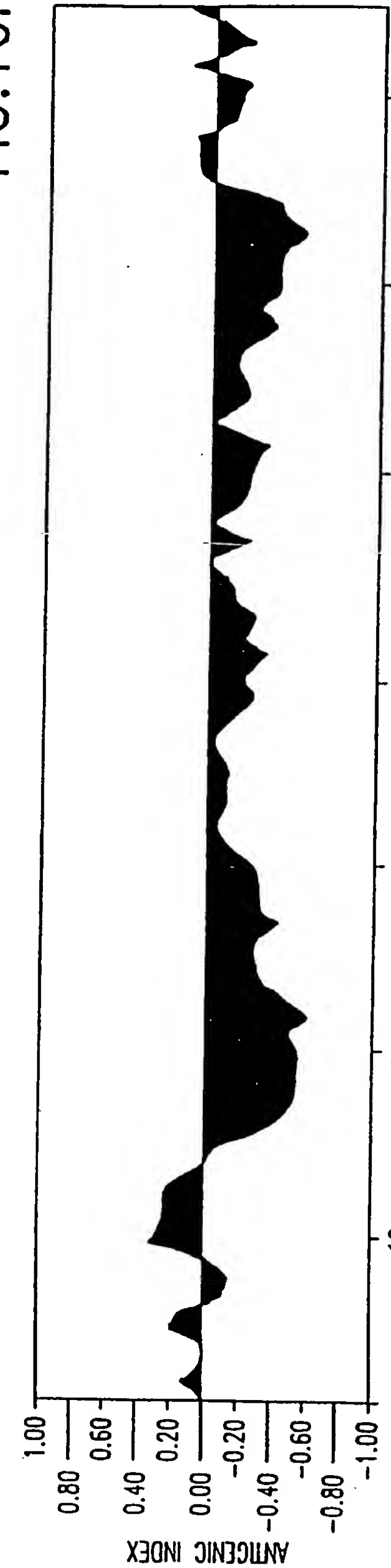


FIG.16Q

AD10-7 SEQUENCE

TTTTTTTTTT GAGATGGAGT TTTCGCTCTT GTTGCCCAGG CTGGAGTGCA ATGGCGCAAT 60
CTCAGCTCAC CGCAACCTCC GCCTCCCGGG TTCAAGCGAT TCTCCTGCCT CAGCCTCCCC 120
AGTAGCTGGG ATTACAGGCA TGTGCACCAC GCTCGGCTAA TTTTGTATTT TTTTTTAGTA 180
GAGATGGAGT TTAACTCCAT GTTGGTCAGG CTGGTCTCGA ACTCCCGACC TCAGATGATC 240
TCCCGTCTCG GCCTGCCCAA AGTGCTGAGA TTACAGGCAT GAGCCACCAT GCCCGGCCTC 300
TGCCTGGCTA ATTTTTGTGG TAGAAACAGG GTTTCACTGA TGTTGCCCAA GCTGGTCTCC 360
TGAGCTCAAG CAGTCCACCT GCCTCAGCCT CCCAAAGTGC TGGGATTACA GGCGTCAGCC 420
GTGCCTGGCC TTTTTATTTT ATTTTTTTTA AGACACAGGT GTACCACTCT TACCCAGGAT 480
GAAGTGCAGT GGTGTGATCA CAGCTCACTG CAGCCTTCAA CTCCTGAGAT CAAGCAATCC 540
TCCTGCCTCA GCCTCCCAAG TAGCTGGGAC CAAAGACATG CACCACTACA CCTGGTAATT 600
TTTATTTTTA TTTTTAATTT TTTGAGACAG AGTCTCACTC TGTCACCCAG GCTGGAGTGC 660
AGTGGCGCAA TCTTGGCTCA CTGCAACCTC TGCCTCCCGG GTTCAAGTTA TTCTCCTGCC 720
CCAGCCTCCT GAGTAGCTGG GACTACAGGC GCCCACCACG CCTAGCTAAT TTTTTTGTAT 780
TTTTAGTAGA GATGGGGTTT CACCATGTTC GCCAGGTTGA TCTTGATCTC TTGACCTTGT 840
GATCTGCCTG CCTCGGCCTA CCCAAAGTGC TGGGATTACA GGTCGTGACT CCACGCCCGGC 900
CTATTTTTAA TTTTTGTTTG TTTGAAATGG AATCTCACTC TGTTACCCAG GTCGGAGTGC 960
AATGGCAAAT CTCGGCTACT CGCAACCTCT GCCTCCCGGG TCAAGCGATT CTCCTGTCTC 1020
AGCCTCCCAA GCAGCTGGGA TTACGGGACC TGCACCACAC CCCGCTAATT TTTGTATTTT 1080
CATTAGAGGC GGGTTTACCA TATTTGTCAG GCTGGGTCTC AAACTCCTGA CCTCAGGTGA 1140
CCCACCTGCC TCAGCCTTCC AAAGTGCTGG GATTACAGGC GTGAGCCACC TCACCCAGCC 1200
GGCTAATTTG GAATAAAAAA TATGTAGCAA TGGGGGTCTG CTATGTTGCC CAGGCTGGTC 1260
TCAAACTTCT GGCTTCAGTC AATCCTTCCA AATGAGCCAC AACACCCAGC CAGTCACATT 1320
TTTTAAACAG TTACATCTTT ATTTTAGTAT ACTAGAAAGT AATACAATAA ACATGTCAAA 1380
C 1381

FIG.16R

AD16c-SEQUENCE

```
CCATTGTTAG GTTGTCTCTT ACCTGTTAAA ATCAGGAGCT GACAAGAAAT GCTTACCACA   60
AAAGGAGAAA TGCCAGTCTA GTTAACAGTC AAGGAGAGAA ATCAGGAAGA TTATGTGGGT  120
GGAAGAAGTA GATGATGTGG CTGATGAGTG AGTGAGTGAG CAAGCCTCCG CCCAGCTGAA  180
GAAGGAGTCA GAACTGCCCT TTGTTCCCAA CTATTTGGCG AACCCCAGCC TTCCCTTTTA  240
TCTATACACC CACAGCAGAG GATTCAGCCC AGATGCAGAA TGGGGGCCCC TCCACACCCC  300
CTGCATCACC CCCTGCAGAT GGCTCACCTC CATTGCTTCC CCCTGGGAAC CTCCCCTGTT  360
AGGGACCTTT CCCCGGGACC ACACCTCTTT GGCACTAGTT CAGAATGGTG ATGTGTCGGC  420
CCCTCTGCCA TACTAGAACA CCAGAAAGAC AAACGGGTGA TGTTTGTCAG CTACAGTGAG  480
TCTAGAGCCG TCCTGTTTTC TTCTGTCCCG TCCCAAGCCA CCATGTCTCT TCGAGCCTCA  540
AAATGGGACG TATGCAGGAC CAGCGCCCAG ATTCCAAGCC ATTTTTCTTC ACTGGAGCAT  600
TTCCATTTAA TATGCAAGAG CTGGTACTCA AGGTGAGAAT TCAGAACCCA TCTCTTCGAG  660
AAAATGATTT CATTGAAATT GAACTGGACC GACAGAGCTC ACCTACCAAG AGTTGCTCAG  720
AGTGTGTTGC TGTGAGCTGG GTGTTAATCC AGATCAAGTG GAGAAGATCA GAAAGTTACC  780
CAATACTCTG TTAAGGAAGG ACAAGGATGT TGCTCGAGTC AAGATTTCAG GACCTGGAAC  840
TGGTTCTGAT GATAGTGAAA ATAATTTTCT GTTCAGAAAT GCTGCATCAC ACTGACTGAA  900
AGGCCTTGCT ATACAGGAGA GCTTCAAAAC TGACTTACTA ATGCAGCAGG GACTTTTATA  960
CTGAGTATAT GACAGTGTGC ATCACCTCTG GGCCAAGGAC AAGCCATGAT CTAAATGCCT 1020
CAGATGCCCG GGCCAGTCTG GTGCACTGCA TAGTATATAC GAACATCATT CTGCCCAAGG 1080
TAGGAAGCCC CATGACCCCC AAGCAGTGGT GTCCACTCTT CCAAGCCTCT TGGTGCACAA 1140
TAAACCTTAT TGCTTGAAGC TTTGAACGAC TGTGAGAATG GTCTGGCGAG GACGAGAACG 1200
TGGAATTATA TGAGTGTCTT TTGTATCCGA GAATGTAGAG AGTTCTCTGA AGACGACGAC 1260
TGAGAGAGAG CGGACGCTAT TTCTAGCCAC TCCTGTTGAC AGTGCACCTG AAGGGCTGGG 1320
ATGCGTTTTT CTTGGTGTTG CATGCTCACA ACTCTGCTGA CATTGGGAAC TTATGAGAGA 1380
GGAAGACTCG GGAAAGCACA GATACTGGAC AGATGGATTC TGGTGTGGGG AAAGCACAGA 1440
TACTGGACAG ATGGTTCTAG TGTGACTTGT GACTGTGAGG TTTCCTATAA CATATTTATA 1500
AATGTTCATC AGGTTCAAAA GTCTATAAGA ATACAGTTCG AGACTGAATT GCTTCGAAAT 1560
ACTTCGATGT TGGGAACCAA AAGAGCTTTC CCTCCCTCAC TTTTTCCTTT GTAACACTCA 1620
TGACTGCTTC TCTGTCTCGA GTCATCTCTG CATTAACTCC CCTTCGTGGT CACTAGAGGG 1680
CTCTCTGATG CTTCTAAGAC ACTGCTTTTT ACATGCCACA CCCACCGCGT AGAGACAGGG 1740
TCTCACTATG TGGCCCAGGC TGGTCTCAAA CTTCTGGCCT TAAGTGATCG TCCTGTCCTT 1800
CGCGCTCGGA AGAAAGTCGT GGGGATTACA GGTGTGAGCC ACCCGCCCAG CCCCTCCCTT 1860
GTGTTTCAAC CAATCGGAAG TGAATTTAAC TAGATGTAGT AACCTTTTTT TTCTTTGACT 1920
TCTAAAAAAG TTACAGTTTA CTAATAAAGT TAAGTCTGGT TCTGTCCTAG AGGAAATAAA 1980
TTCACTATTA ATTCATGTCT TAAGTTACTT GGGTTAAAAC ACTTTCAGCC ACCCAGATTA 2040
ATTAAAGTGG AGCAGTGGAG CCCCTGGCTG GGGAGATGGG CCTCCAGAGG AGCAGCTGCA 2100
GGCATGTTCT GGCTACACAG AGGCAAGCAA GGGACTGGTG TCTCTGGTGA GAGGTGGGTT 2160
TGATGTATCT CTGTCCTATG CTGGTCTCTC TTCTCCTTTA TAAATCCTCC TGTGGTCACT 2220
GACTATCGTA TCGCAGTGAT CAGACTGCAC ATAGTACGGT TAGGCTGAGC TTAATGTCTT 2280
AATCATGTCA TTCGAGAGAA GACACGTTTT GATTCATGCT TTGTGTAATT AATCAATCAA 2340
GGATTCTTTT TTTAGCTTTG TTGACGTGTA ATTCACCCCT CCTCCTCCAC TGCATATTTA 2400
AAGCATGTGT TCACACTGTG TGTATACATT CACTGCGATT TTTTCGTTTG CTGCATTGCT 2460
TGGACTGTTC ATAACATCAC AAGTATTATT CAAATAAAAT ATTAACTGAC CGAAAAAAAA 2520
```

FIG.16S

```
H REG GENE                5    10    15    20    25    30    35    40    45    50
[ 220 ] ------------GA AtTCC TGggC TCAaG TGATC CtCtC atgTC AGtCT CCCAA AGTGC TGGGA>
AD2-283             GA ACTCC TGACC TCAGG TGATC CGCCC GCCTC AGCCT CCCAA AGTGC TGGGA

H REG GENE   55    60    65       70    75    80       85      90    95   100
[ 220 ]    TgACA gGCtT G-AG- CC--A -CCAc ACcAg gcccA -TC-- AtCa- G--tT ttTaT A-taA>
AD2-283    TTACA AGCGT GCAGA CCGCA CCCAG ACGAT TTTAA TTCTT AACNT GCCAT CCTCT ACCCA

H REG GENE  105   110   115   120   125  130   135   140
[ 220 ]    AGaAa AaAaa ACcTT AaaaT tgtTA gCAAA tacta tGACA>
AD2-283    AGTAC AGACC ACTTT AGTGT CCCTA TCAAA GGGGG AGACA
```

FIG.17

```
AD2 SP6F    110     115    120   125   130   135   140   145   150
[ 62 ]    ---AA ttC-t C-CtG cCTCA GCCtc gtGag ccGct GGgAT TACAG GcG>
EXONI        AA GCCAA CTCAG ACTCA GCCAA CAGGT AAGTG GGCAT TACAG GAG


RAT PTP    605
[ 144 ]   <ACTC
AD2-2 T7   ACTC

RAT PTP   660   655   650  645    640   635   630   625      620   615   610
[ 144 ]   <tcT-a ggaAg aGggg GTTGA C---t tTGCT TTTGA taGaT GGT-c TagT- TTCac TTttg
AD2-2 T7   AGTGT TTTAT TGCTA GTTGA CAAAG CTGCT TTTGA ATGCT GGTGG TTCTA TTCCT TTGAC

RAT PTP      710   705   700   695    690   685    680    675    670   665
[ 144 ]   <aCA-T aCAAt AaTGg aGatA -aaaa TaCcA T-A-G GgCAg T--GA GGcA- AgaaT GTTtG
AD2-2 T7   TCATT TCAAA ATTGC TGCCA GTGTT TTCAA TGATG GACAA TCAGA GGGAT ATGCT GTTAG

RAT PTP                               745 740 735 730 725 720 715
[ 144 ]                                <GTg A-Agg taTTT TTatT TaaaT gTgca gggTT
AD2-2 T7                                GTA ATACT CCTTT TTTCT TCTTT CTTTT TTTTT


                                     5
             --------------------FVf cLII>
HPTPAA                           FVA SLIK
```

FIG.17A

```
1-9aT7-3 3 140   145   150   155   160   165   170   175   180   185   190   195
[ 206 ]   ACcAC GCCCc GCTAA TtTTT GTATT TTTAG TAGAG AcaGG GTTTC aCCgT GTTgg cCAGG>
AD2-283   ACAAC GCCCA GCTAA TATTT GTATT TTTAG TAGAG ATGGG GTTTC TCCAT GTTCA TCAGG

1-9a-T72 3  15    20    25    30    35    40    45    50    55    60    65    70
[ 260 ]   CTGGT cTgaA AtTCC TGggC TgAaG TGATC CtCCa GtCTt gGCCT CCCAA AGTGC TGGGA>
AD2-283   CTGGT GTCGA ACTCC TGACC TCAGG TGATC CGCCC GCCTC AGCCT CCCAA AGTGC TGGGA

1-9a-T72 3  140    145   150   155   160
[ 260 ]   AG-AC AcA-t A-Tag AtTGa gaC-A gaAAA>
AD2-283   AGTAC AGACC ACTTT AGTGT CCCTA TCAAA
```

FIG.17B

```
AD16c-SP6                           5    10    15    20    25    30
[ 344 ]                         —AGA- TcTCg CTC-T G-Tca CCCAG GCTGa AGTGC>
AD2-2 SP6                        AGAG TTTCA CTCTT GCTTG CCCAG GCTGG AGTGC

AD2-2 SP6    35    40    45    50    55    60    65    70    75    80    85
[ 344 ]    AgTGG CcCAA TCtcG GCTCA CTGCg AgCTC C-aCC TCCCG gGtTC Acttc aTTCT CCTGC>
AD2-2 SP6  AATGG CACAA TCCTG GCTCA CTGCA ACCTC CGCCC TCCCG AGCTC AAGAA CTTCT CCTGC

AD16c-SP6  100   105   110   115   120   125   130   135   140145   150   155   160
[ 344 ]    CTCAG CCTC- TGAGt aGCTG GGAcT ACAGG CGCcC aCCAC AcGCc gCTAA TtTTT GTATT>
AD2-2 SP6  CTCAG CCTCG TGAGC CGCTG GGATT ACAGG CGCGC GCCAC AAGCG ACTAA TATTT GTATT

AD16c-SP6
[ 344 ]    TTTGT AG>
AD2-2 SP6  TTTGT AG

AD16c-SP6   140   145   150   155   160   165   170   175   180   185   190   195
[ 206 ]    ACcAC GCCCc GCTAA TtTTT GTATT TTTAG TAGAG AcaGG GTTTC aCCgT GTTgg cCAGG>
AD2-283    ACAAC GCCCA GCTAA TATTT GTATT TTTAG TAGAG ATGGG GTTTC TCCAT GTTCA TCAGG

AD16c-SP6   200    205   210
[ 206 ]    aTGcT —CGA tCTCC TGA>
AD2-283    CTGGT GTCGA ACTCC TGA
```

FIG.17C

```
H REG GENE   3610  3615   3620  3625  3630    3635  3640 3645  3650  3655  3660
[ 118 ]      —CC CC-aa gC-aG tGTta aTcct GG-cT A—CT GtgTG AGcTG AccTC AagcA CaGGt>
AD3-4           CC CCTGT TCTTG GGTGG GTTTG GGTAT ATTCT GGTTG AGATG ATATC ATTTA CGGGG

H REG GENE      3670  3675   3680  3685   3690  3695 3700  3705  3710  3715  3720
[ 118 ]      GAAGG Cagag aaTcc A-Tcc aCC-T gTTTC TgTTc TCCcT gCtTA gctcc AGGga TgGAA>
AD3-4        GAAGG CGCTT TGTGA AGTAG GCCTT ATTTC TCTTG TCCTT TCGTA CAGGG AGGAT TTGAA

H REG GENE  3725  3730  3735   3740  3745  3750   3755   3760  3765  3770
[ 118 ]      cTgGg Actgg GaT-a gAgga aaG-g TGAAC TC-cT CA-tT aagga aATgG aTG>
AD3-4        GTAGT AGAAC GCTGT TACTC CGGTC TGAAC TCAGT CACGT GGCTT TATCG TTG
```

FIG.18

```
WP03-5 T7      5        15    20    25   30    35    40    45    50
[ 90 ]       GATCC aAGCT acGTA -CgcG TgcAT GCAcg tCaTa gcTcT TCTAT AGTGT CAC>
AD3-4 221    GATCC GAGCT CGGTA CCAAG TTGAT GCATA GCTTG AGTAT TCTAT AGTGT CAC

18-4T7       155   150   145      140   135   130   125   120   115   110   105
[ 362 ]     <gTATg GgCcc gATAg —c-t TAT-t TAgcC TTTAG AGCAC ACTGG CgGCC GTTAC TAGTG
AD3-4 221    ATATA GACAA TATAA CAATA TATTG TATAC TTTAG AGCAC ACTGG CAGCC GTTAC TAGTG

18-4T7       100   95    90    85   80    75    70    65    60    55    50    45
[ 362 ]     <GATCC GAGCT CGGTA CCAAc TTGAT GCATA GCTTG AGTAT TCTAT AGTGT CACct -aAaT
AD3-4 221    GATCC GAGCT CGGTA CCAAG TTGAT GCATA GCTTG AGTAT TCTAT AGTGT CACTA ATAGT
```

FIG.18A

```
G2A-EP T7             20    25     30    35       40    45    50          55
[ 148 ]    ----------CTT AaTA- gAtAg ctaCT TA--- AAAta AcTTA CaC-A cT--- GTtTt>
AD3-4 SPF             CTT ACTAC CAGAC AACCT TAGCC AAACC ATTTA CCCAA ATAAA GTATA

G2A-EP T7  60    65    70    75    80    85    90    95   100   105     110
[ 148 ]    aGaG- T-G-- cTTGA AAaCT atCtg AtcAG AcATA GTAtt GaAAc cAAtG A--At AcATT>
AD3-4 SPF  GCCGA TAGAA ATTGA AACCT GGCGC AATAG ATATA GTACC GCAAG GAAAG ATGAA AAATT

G2A-EP T7 115   120   125    130   135
[ 148 ]    AT-At aAAG- -TAA- A-gGa AAGGA -gAA>
AD3-4 SPF  ATAAC CAAGC ATAAT ATAGC AAGGA CTAA

AD3-4                          215   210  205     200   195   1901185  180
[ 182 ]    -------------------<CT ATAaA GgTcG TTG-- TcaaC gaTaA AGCAc GtGAc
H REG GENE                      CT ATAGA GATTG TTGAT TTGCC TCTTA AGCAA GAGAT

AD3-4        175     170  165   160   155   150   145   140   135     125
[ 182 ]    <TgAgt tCAG- aCcGg A-Gta aCAG- CgttC TacTA CTTCA -aaTc cTC-C CTGCg aaAgG
H REG GENE  TCATT GCAGC TCAGC ATGGC TCAGA CCAGC TCATA CTTCA TGCTG ATCTC CTGCC TGATG

AD3-4      120   115   110   105    95    90    85    80    75    70    65    60
[ 182 ]    <caaga Gaaat AagGC CtAct TAAGc gC-CT TccCC cgtAA atgAt atcaT CTcaa CCaGA
H REG GENE  TTTCT GTCTC AGAGC CAAGG TAAGA TCTCT TTTCC ACCAA CCAAC TCTTT CTAGC CCTGA

AD3-4         55    50    45   40    35    30    25    20    15    10
[ 182 ]    <AtA-T aCcCa aaccC CCCAA GaAcA gGGGa ggAaa aGAAA AAAAA AAAAA
H REG GENE  AGACT TCACT CTATC CCCAA GCATA CGGGT CTACT TGAAA AAAAA AAAAA
```

FIG.18B

```
AD2-2 T7                       535   530   525   520   515   510   505   500   495
[ 110 ]   ---------------<CAGAA AAcTa GCtAc aC-AA tgcAt TGGTC TATcA tgtta aAAcg
AD4-4 T7F                     CAGAA AAATT GCCAA GCTAA ACTAA TGGTC TATAA AAGCG TAATT

AD2-2 T7    490         485   480   475   470   465         460
[ 110 ]    <TGCAT -T--a GaCAc AAA-T ACAaA aacCA TG--A -AacA aGCcA
AD4-4 T7F   TGCAT GTGTG GGCAT AAACT ACAGA GCTCA TGCTA GAGTA TGCAA


1-9a                       145   140     135   130   125    120   115    110
[ 142 ]   -------------<AA -TTGg gTaC- -Cggg cccCc cCTA- gAggT cgAcG gTAT- cGA-T
AD4-4 T7F                   AA GTTGA TTGCT TCAAA TTTCT GCTAC TAACT TCAAG CTATG GGAGT

1-9a        105   100   95    90    85    80          75    70    65    60    55    50
[ 142 ]   <aaGct tGTAt cgAaT TccGG AcTTT gcTT- ---TT gGTTT TCcTT Tcctg TGaaA AgGtT
AD4-4 T7F   TTGGC AGTAG TCACT TGAGG ATTTT TTTTC CAATT CGTTT TCATT TTTGT TGTTA AAGCT

1-9a              45    40          35    30    25          20    15    10
[ 142 ]   <gGTtt TaaAG TGAG- -AtAc ActTT tCC-- GtagA ACaAg TGtTC TAT
AD4-4 T7F   CGTAC TTCAG TGAGA CAGAA AAATT GCCAA GCTAA ACTAA TGGTC TAT
```

FIG.19

```
H REG GENE                        5     10    15    20    25    30    35    40   45
[ 278 ]-------------------g AATTC CTGGG CTcAA GTGAT CCTCt caTgT caGtC TCCCA AAGTG>
AD16C-T7-A                A AATTC CTGGG CTGAA GTGAT CCTCC AGTCT TGGCC TCCCA AAGTG
                          /

H REG GENE   50    55    60    65    70    75    80    85    90    95    100   105
[ 278 ]    CTGGG ATgAC AGGCt TGAGC cACca caCCa gcCCa TcATc AgTtt TTaTA tAaaG -aaaa>
AD16C-T7-A CTGGG ATTAC AGGCA TGAGC TACTG AGCCT AGCCT TAATG ATTAA TTTTA GAGTG ATGGC

H REG GENE   110   115    120   125   130     140   145   150   155   160
[ 278 ]    aaaaA CCT-t A--aA Attgt TA-gG caaAt AatGA cAAaT TgtAa ta-ta tattc ttACa>
AD16C-T7-A TTGTA CCTTC AAGCA ACATA TAGAG TTGAG ACAGA AAATT TCCAT CGTCC CGAGA AAACT

H REG GENE 165   170   175   180   185  190   195   200   205   210   215   220
[ 278 ]    tTtCa -gATt tttat ttTtT aaaCT GatAA GAatt gatTA aTAAa TAaAA tTtaG tAT-->
AD16C-T7-A GTGCT GCATG GGCCC CGTGT GCCCT GTGAA GATCG CCCTA TTAAC TATAA ATGGG CATTG

H REG GENE       225   230   235
[ 278 ]    t-aA- -tcTG t-ctt TTaA>
AD16C-T7-A CACAT GGTTG CCAGC TTCA
```

FIG.20

```
AD16C                                                     5     10    15    20
[ 266 ]  ----------------------------------------------oACgt TtcGA oCCTo tCgTg>
Human-PTP                                             TACTG TGTGA GCCTG ACCTC

AD16C            25    30    35    40    45       50    55    60    65     70
[ 266 ]   AAG-c -ccGA TTttA GAgtT ootAc -ggGT -gC-- tTcAA GggA- ocggg gCToT --go->
Human-PTP AAGCA CAGGA TTCCA GAAAT GGAAG GATGT GCCTT GTGAA GACAA GTTCT CCTTT GTCTG

AD16C          75      80    85       90    95   100   105   110115120   125   130
[ 266 ]   gAAGT -tttc tACgg GgoGC --oTG GAAAt TttcT GTCTc oAtoT GtgCt tGoAg gTACA>
Human-PTP CAAGT TCAAA AACTA GAGGC AGCTG GAAAA TACAT GTCTA GAACT GATCC AGCAA TTACA

AD16C       135  140   145   150   155    160   165   170   175 180    185   190
[ 266 ]   ACcGt oTCtA AAATT AAtCo tt-oo ggCTo ggCtc ogtAg CTCtg CCTGt -o-oT CcCog>
Human-PTP ACGGA GTCAA AAATT AAACC GGACC ATCTC TCCAA CTCAA CTCAA CCTGG ACACT CTCTT

AD16C         195   200   205      210   215   220   225    230   235    240
[ 266 ]   CoC-t tTcgG gogGC Coo-- gAcTg goggA TcocT TcAg- ccCog gAo-t TTcoG AcgCc>
Human-PTP CTCTG CTGAG TTTGC CTTGT TAATC TTCAA TAGTT TTACC TACCC CAGTC TTTGG AACCT

AD16C-T7-A                                          205
[ 33 ]   -----------------------------------------VPCE Dr>
HPTPAA                                              VPCE DK

RPTP AA    115   120
[ 33 ]     SgSLf LyKsW D>
Translatio SSSLG LPKCW D
```

FIG.20A

ALIGNMENT OF AD16C-SP6 cDNA WITH AD2-2 SP6 cDNA

```
AD2-2 SP6   40    45          50    55    60    65    70    75    80    85    90
 [ 362 ]    CTtGC T-TG- --CCC AGGCT GgAGT GCAaT GGCaC AATCc tGGCT CACTG CaAcC TCCcC>
AD16C-SP6-  CTCGC TCTGT CACCC AGGCT GAAGT GCAGT GGCCC AATCT CGGCT CACTG CGAGC TCCAC

AD2-2 SP6 95   100   105   110   115   120   125   130         135   140   145
 [ 362 ]    CTCCC GaGcT CAaga acTTC TCCTG CCTCA --GCC TC-G- ---TG AGccG CTGGG AtTAC>
AD16C-SP6-  CTCCC GGGTT CACTT CATTC TCCTG CCTCA CTGCC TCAGC CTCTG AGTAG CTGGG ACTAC

AD2-2 SP6   150   155   160         165   170   175   180   185
 [ 362 ]    AGGCG CgCgC CA-CA aG--- -CgaC TAA-T aTTTG TATTT TT-GT AG>
AD16C-SP6-  AGGCG CCCAC CACCA CGTCC CCTGC TAATT TTTTG TATTT TTAGT AG

AD2-283       50    55    60    65    70    75    80 85     90    95   100   105
 [ 374 ]    CCaTG TTcat CAGGc TGGTg TCGAa CTCCT GACCT CGTGA TCCGC CCGCC TcaGC CtCCC>
AD16C-SP6-  CCGTG TTGGC CAGGA TGGTC TCGAT CTCCT GACCT CGTGA TCCGC CCGCC TTGGC CACCC

AD2-283     110   115   120   125   130
 [ 374 ]    AAAGt G-cTG GGATT ACAaG CGTGC>
AD16C-SP6-  AAAGA GTTTG GGATT ACAGG CGTGC
```

FIG.20B

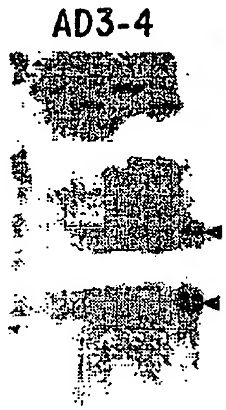


FIG. 21A

FIG. 21B

FIG. 21C

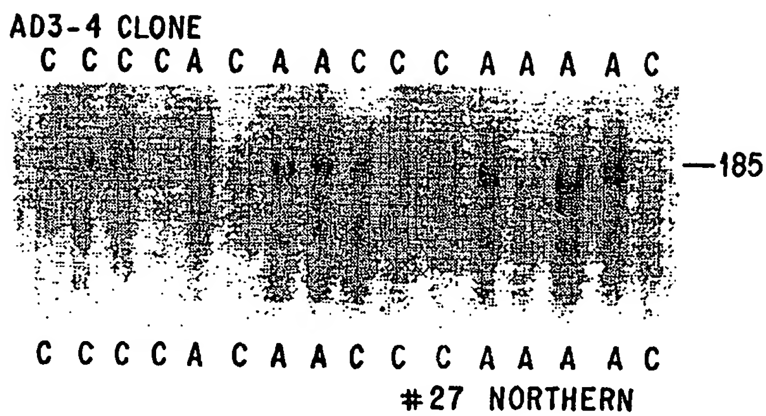


FIG. 21D

G2-2Psl-M13F SEQUENCE

Sequence Range: 1 to 251

```
TGCAG CAATG GCAAC AACGT CTGCA AACTA TTAAC TGGCG AACTA CTTAC TCTAG CTTCC  60
CGGCA ACAAT TAATA GACTG GATGG AGGCG GATAA AGTTG CAGGA CCACT TCTGC GCTCG 120
GCCCT TCCGG CTGGC TGGTT TATTG CTGAT AAATC TGGAG CCGGT GAGC GTGGG TCTCG 180
CGTAT CATTC GAGCA CTGGG GCCAG ATGGT AAGCC CTCCG TATCG TAGTT ATCTC ACAGC 240
AGGGA GTCAG G                                                           251
```

FIG.22

G2-2Psl-M13R SEQUENCE

Sequence Range: 1 to 242

```
TGCAG GACCG GGGAG GCACG ATGGC CGCTT TGGTC CGGAT CTTTG TGAGG AACCT TACTT  60
CTGTG GTGTG ACATA ATTGG ACAAA CTACC TACAG AGATT TAAAG CTCTA AGGAA ATATA 120
AAATT TTTAA GTGTA TAATG TGTTA AACTA CTGAT TCTAA TTGTT TGTGT ATTTT AGATT 180
CCAAC CCTAT GGAAC CTGAT GAATG GGAGC CAGTG GTGGA ATGCC TTTAA TGAGG AAACC 240
TG                                                                      242
```

FIG.22A

G2-2Psl1-EcoR1-M13F SEQUENCE

Sequence Range: 1 to 208

```
TGCAG CAATC TTTCT TATAT ACATG CTTAA TAGAT AGCTA CTTAA AATAA CTTAC ACACG  60
TTTTA GAGTT GCTTG AAAAC TATCT GATCA AGACA TAGTA ATTGA AACCA ATGAA TACAT 120
TATAT AAAGT AAAGG AAAGG AGAAG AGAGG AAAGG GACGG GAAGA GGAGA GGGAG GGACA 180
AGCGA GAAAG GAAAG GGAAG GGAGA AAA                                       208
```

FIG.22B

Gen2-2Pst1-EcoR1-M13R SEQUENCE

Sequence Range: 1 to 152

```
CTCAC TAAAG GGATC AAGGA ATAAT TTTGA ATTTC AAGTC TTACA TTTAA TAAAT ACATT  60
CATAA GGCTA TAACT ACCAT ACGTT GTGAT TTCTC TGATT AATTT AAAAA TAAAT TAAAA 120
CCTGG AAAGA ATTTT ACCAT TCTAG GAAGC CA                                 152
```

FIG.22C

G2-2Pst1-EcoR1-T7 SEQUENCE

Sequence Range: 1 to 338

```
AATCT ATCTT ATATA CATGC TTAAT AGATA GCTAC TTAAA ATAAC TTACA CACGT TTTAG  60
AGTTG CTTGA AAACT ATCTG ATCAA GACAT AGTAA TTGAA ACCAA TGAAT ACATT ATATA 120
AAGTA AAGGA AAGGA GAAGA GAGGA AAGGA GGGGA GAGGA GAGGA GGACA AGCGA GAAAA 180
GGAAG GGAAG GGAGA AAAAG GGGGA AAGGG AGGTA GAGAG AGAGA GAAAA AGTGC TGGTC 240
ATATA GTAAG TGTAC ATTTT AACTT TTTAA GAAAC TACCC TACTC TATTC CAGAG TGATT 300
GTACA TGTGC ATTTT ACTGC ATTAT AGAGA TCATT TTC                          338
```

FIG.22D

G5dPst-M13R SEQUENCE

Sequence Range: 1 to 169

```
TGCAG GAGTG GGGAG GCACG ATGGC CGCTT TGGTC CGGAT CTTTG TGAAG GAACC TTACT  60
TCTGT GTGTG ACATA ATTGG ACAAA CTACC TACAG AGATT TAAAC GTCTA AGGTA AATAT 120
AAAAT TTTTA GTGTA TAGGT TAAAC TACTG ATTCT AATGT TGTGT ATTT             169
```

FIG.22E

G5d Pst-T71 SEQUENCE

Sequence Range: 1 to 209

CCCCG GGCTG CAGCA ATGGC AACAA CGTCT GCAAA CTATT AACTG GCGAA CTCAT TCATC 60
TAGCT TCCCG GCAAC AATTA ATGAC TGGAT GGAGG CGGAT AAAGT TGCAG GACCA CTTCT 120
CGCGT GGCCC TTCCG GCTGG CTGGT TTATT GCTGA TAATT GAGCG TGCGA GTGGC TCGCG 180
TATCA TTCGC GACAT GGGCC AGTAG GTAC 209

FIG.22F

G5dPst1-EcoR1-SP SEQUENCE

Sequence Range: 1 to 272

CTTGC CCTTC ATGGA GTCAT ACACC CGATC AGCAA AATCC AGGGG CTTGT TCTGA ATGCA 60
CTGAA CCAGG TTCAG GAAAG CATTT TCCAG GTCTC CTTTA ACCTC TTTCC TGATG CTTTC 120
CAACA TGTCA TAAGG GCTGT AACTC TTGTA CCTAT CAAAT ACTTT CTGGA GGTGG GGACA 180
CGCTC CGCTC GGTCA TGATG CTGAT CCACT TGGGA ACATC AGTTC TTTCC TCTTC ACTCC 240
AGCTG CATAG AGATC CGAGG ACTCT TGGTC AA 272

FIG.22G

G5dPst1-EcoR1-t7 SEQUENCE

Sequence Range: 1 to 278

ACGGC CCAGC TTCCT TCAAA ATGTC TACTG TTCAC GAAAT CCTGT GCAAG CTCAG CTTGG 60
AGGGT GATCA CTCTA CACCC CCAAG TGCAT ATGGG TCTGT CAAAG CCTAT ACTAA CTTTG 120
ATGCT GAGCG GGATG CTTTG AACAT TGAAA CAGCC ATCAA GACCA AAGGT GTGGA TGAGG 180
TCACC ATTGT CAACA TTTTG ACCAA CCGCA GCAAT GACAC GAGAC AGGAT ATTGC CTTCG 240
CCTAC CAGAG AAGGA CCAAA AAAGG AACTT GCATC ACA 278

FIG.22H

ALIGNMENT OF G2-2PstI with HUMAN REG GENE (1)

```
H REG GENE      3405  3410    3415  3420  3425   3430  3435    3440  3445  3450  3455
[ 228 ]         ---AG CAATa GCAA- -AgGa aaGgA AAC-A aTAtt TaGC- AA-gg tTTAt TCTtc CTTtg>
G2-2Pst-M13F    AG CAATG GCAAC AACGT CTGCA AACTA TTAAC TGGCG AACTA CTTAC TCTAG CTTCC

H REG GENE          34653470  3475  3480  3485  3490  3495   3500   3505  3510
[ 228 ]         tGtCA gCAtT TctgA GtgTG cAcac AGGCc cAgtg A-TTc CAtG- -tAtT TtTGa G-T-G>
G2-2Pst-M13F    CGGCA ACAAT TAATA GACTG GATGG AGGCG GATAA AGTTG CAGGA CCACT TCTGC GCTCG

H REG GEN3515     3520  35253530    3535  3540     3550  3555  3563565     3575  3580
[ 228 ]         aCCac TgCct CTGtC TGG-c ccTTc CccAT AgAaC cGccG CtGGT gGAGC GTGGG TCcCt>
G2-2Pst-M13F    GCCCT TCCGG CTGGC TGGTT TATTG CTGAT AAATC TGGAG CCGGT CGAGC GTGGG TCTCG

EXON                                                 20    25      30    35    40
[ 124 ]         -------------------------------------C TGGca CtGG- aG-ca GTGGG TCcCt>
G2-2Pst-N13F                                         C TGGAG CCGGT CGAGC GTGGG TCTCG

H REG GENE      3585  3590  3595  3600    3605   3610  3615    3620   3625  3630
[ 228 ]         gGTcT CcTaC aAGtc CTGGG G-CA- tTGG- -AGCC CcaaG cA--G T-GTT A-aTC ctgGC>
G2-2Pst-M13F    CGTAT CATTC GAG Q CTGGG GCCAG ATGGT AAGCC CTCCG TATCG TAGTT ATCTC ACAGC

EXON5           45    50    55    60     65     70     75    80     85    90    95
[ 124 ]         gGTcT CcTaC aAGtc CTGGG G-CA- tTGG- -AGCC CcaaG cA--G T-GTT A-aTC ctgGC>
G2-2Pst-M13F    CGTAT CATTC GAG Q CTGGG GCCAG ATGGT AAGCC CTCCG TATCG TAGTT ATCTC ACAGC

H REG GENE3635    3640
[ 228 ]         ActGt GTgAG>
G2-2Pst-M13F    AGGGA GTCAG

EXON5            100   105
[ 124 ]         ActGt GTgAG>
G2-2Pst-Ma3F    AGGGA GTCAG
```

FIG.23

ALIGNMENT OF G2-2Pst with HUMAN REG GENE (2)

```
H REG GENE   3155  3160  3165   3170  3175  3180  3185  3195       3200
[ 194 ]    ——AG GAGac ttGtG GtA-a AaatC tGCTg ctGTa CtGcT CaTT- TG-GG AACCT TA-gT>
G2-2Pst-M13R AG GAGCG GGGAG GCACG ATGGC CGCTT TGGTC CGGAT CTTTG TGAGG AACCT TACTT

H REG GEN3210  3215  3220  3225   3230  3235  3240  3245  3250  3255  3260   3265
[ 194 ]      aTact aaaTa AtATA A-Tat AtcAA CaACt aAtgG tcAgc cAAtG CTaTg ctG-g ATATg>
G2-2Pst-M13RCTGTG GTGTG ACATA ATTGG ACAAA CTACC TACAG AGATT TAAAG CTCTA AGGAA ATATA

H REG GENE 3270  3275   3280  3285  3290  3295   3300  3305   3310  3315  3320
[ 194 ]      AgggT ccT-g GgccA cAAaG acaaA AAaT- CaGga aCcAc TT-TT TaaGT gagaT ActTT>
G2-2Pst-M13RAAATT TTTAA GTGTA TAATG TGTTA AACTA CTGAT TCTAA TTGTT TGTGT ATTTT AGATT

H REG GEN3325  3330   3335  3340  3345  3350  3355  3360  3365  3370  3375
[ 194 ]      gggtC tCTgT -cAAa tTcAT aAcac ttAtt tctTG GTGGA ATaCa gTTAA TGAG>
G2-2Pst-M13RCCAAC CCTAT GGAAC CTGAT GAATG GGAGC CAGTG GTGGA ATGCC TTTAA TGAG


G2-2Pst-Ma3F       150   155   160         165   170    175   180   185   190   195
[ 130 ]          ————TA aAtCt GgaGC cGG—— -TcGA GC-GT GGGTC tCgcG TaTCa TtCgA GcaCT>
EXON5                TA GAACC GCCGC TGGCA CTGGA GCAGT GGGTC CCTGG TCTCC TACAA GTCCT

G2-2pst-M13F 200      205      210   215   220   225   230    235   240   245   250
[ 130 ]      GGGGC ——caG A-tgg tAAGC ccTcc gtATC gTaGt TA-Tc TcacA GCagG gagTC AgG>
EXON5        GGGGC ATTGG AGCCC CAAGC AGTGT TAATC CTGGC TACTG TGTGA GCCTG ACCTC AAG
```

FIG.23A

ALIGNMENT OF G2-2PST-ECOR1-T7 WITH HUMAN REG GENE

```
H REG GENE                                   335   340   345   350   355   360   365
[ 132 ]    ---------------------------------AT AGATA tCTAC TTtAt tcgAt TTAaA ttCTG -TTTA>
G2-2PST-ECOR1 T7                            AT AGATA GCTAC TTAAA ATAAC TTACA CACTG TTTTA

H REG GENE     370   375   380   385        390   395   400   405   410   415
[ 132 ]    --GTa tTTtA ttA-T ATtTt gTtA- A--T- ccAtT TG-tc CCAAT tcATA tAcT- TAT>
G2A-EP T7  GAGTG CTTGA AAACT ATCTG ATCAG ACATA GTAAT TGAAA CCAAT GAATA CATTA TAT
```

```
RAT PTP                                                           740   735
[ 82 ]   ------------------------------------------------------<TqAAq qtATT
G2-2PST-ECOR1-M1                                                     TAAAT
ACATT

RAT PTP           730   725   720   715     710   705   700   695   690   685
[ 82 ]      <--Ttt atTA -AAtg tgCA- ggGTT -acA- TaCaa TaATg gAgaT AAAAA TAcca TAggg
Gen2aEP-Ma  CATAA GGCTA TAACT ACCAT ACGTT GTGAT TTCTC TGATT AATTT AAAAA TAAAT TAAAA

RAT PTP           68675  670   665      660   655
[ 82 ]            <CgaGG cAAGA ATgTT ---tg TCTAG GAAG
G2-2PST-ECOR1-MI  CCTGG AAAGA ATTTT ACCAT TCTAG GAAG
```

FIG.23B